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A Novel Anti-Cancer Stem Cells Compound Optimized from the Natural Symplostatin 4 Scaffold Inhibits Wnt/β-Catenin Signaling Pathway

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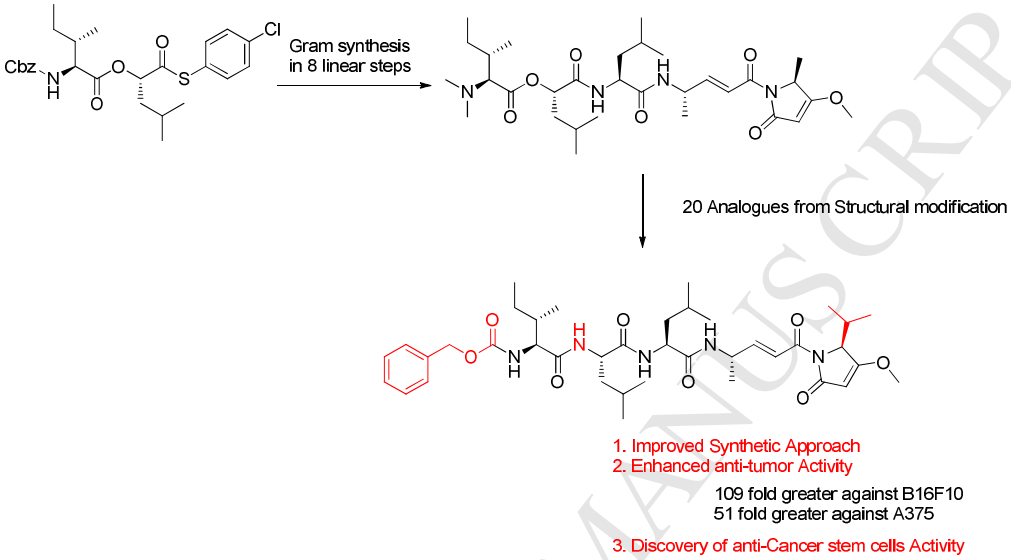

A Novel Anti-Cancer Stem Cells Compound Optimized from the Natural Symplostatin 4 Scaffold Inhibits Wnt/β-Catenin Signaling Pathway

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Hightlights:
Cancer Stem Cells
Total Synthesis
Symplostatin 4
Depsipeptides

Abstract

Cancer stem cells (CSCs) are responsible for carcinogenesis, cancer progression, relapse, metastasis and drug resistance. Therefore, the development of drug molecules targeting CSCs plays a vital role in medicinal researching field. However, there are extremely rare molecules that selectively ablate CSCs. The research and development of drugs targeting CSCs is limited due to a lack of anti-CSCs lead compounds. In this study, an anti-CSCs lead compound **35b** was discovered, which was derived from the natural chemical scaffold of Symplostatin 4. This compound exhibited a significantly suppressive effect on tumor growth both *in vitro* and *in vivo*. Additionally, **35b** could significantly reduce the number of melanoma tumor spheres and decrease the percentage of $ALDH^+$ melanoma cells. Further mechanism study illustrated that compound **35b** could eliminate the melanoma CSCs by efficiently blocking Wnt/β-catenin signaling pathway. Collectively, our findings would provide a novel chemical scaffold and alternative idea of molecular design for development of anti-CSCs drugs.

INTRODUCTION

Medicinal studies on natural products exert a crucial impact upon both chemical biology and drug discovery. More than half of all clinical medicines have their origin from natural sources, especially anti-cancer drugs. However, the miscellaneous chemical structures and moderate activities of natural molecules have severely impeded the progress of novel drug development. Synthesis and optimization of natural product scaffolds have not only brought an effective solution to drug discovery, but also prepared novel chemical structures with improved efficacies.

Cancer stem cells (CSCs) are defined as a type of rare cancer cells with embryonic or somatic stem cell-like properties, including the ability of self-renewal and multipotent differentiation

potential [1]. Although the origin of CSCs has not been well understood, the presence of these cells has been found in most malignancies, such as hematological malignancy, lung cancer, breast cancer, gliomas, ovarian cancer and melanoma [2]. Of note, an avalanche of evidence has demonstrated that CSCs are critically involved in tumorigenesis, cancer invasion, metastasis, tumor recurrence and therapeutic resistance [3]. Hence, CSCs are hypothesized to be the progenitor cells for tumor recurrence at primary or distant locations after appropriate and standard cancer therapy. However, drugs targeting common cancer cells generally have no effects on CSCs, due to the special characteristics of CSCs. [4] Thus, it is urgent to probe into novel therapeutic strategies targeting CSCs.

Wnt/β-catenin signaling pathway, a canonical Wnt pathway, is considered to be an important regulator for the maintenance of self-renewal of CSCs. Constitutive activation of Wnt/β-catenin signaling pathway in CSCs strongly contributes to cancer proliferation, relapse and metastasis. [5] In brief, in activated Wnt signaling pathway, activated disheveled homologue (Dvl) phosphoprotein, a downstream effector of the receptor, inhibits Axin-mediated phosphorylation and degradation of β-catenin, resulting in its accumulation in cytoplasm. Then, β-catenin translocates into nucleus and interacts with T-cell factor/lymphoid-enhancing factor (TCF/LEF) to activate the transcription of downstream targeted genes, which subsequently regulate cell proliferation and survival. [6] In diverse types of malignancies, dysregulation of the expression of Wnt, Frizzled receptors, Axin, and mutation of APC or β-catenin has been proved to induce the persistent activation of Wnt/β-catenin signaling pathway. [7] Therefore, targeting these key regulators to control the self-renewal and differentiation of CSCs is a novel strategy to overcome CSCs-associated tumorigenesis for cancer treatment.

Symplostatin 4 (also known as Gallinamide A), is a natural depsipeptide independently isolated from the *Symploca* genus in Key Largo, Florida and from *Schizothrix* species of cyanobacteria from the Carribean Coast of Panama. [8] Firstly, Symplostatin 4 was found to be one of the most potent anti-malaria depsipeptides, as it strongly exhibited the activity of cultured *P. falciparum in vitro*, with an IC_{50} of 50 nM. More importantly, Symplostatin 4 did not exhibit any hemolytic activity against red blood cells, nor did it inhibit the proteasome. These above properties have made this natural product as an ideal leading compound for development of new type anti-malaria drugs. The total synthesis of this molecule has been reported previously by Payne's group [9] and Kaiser's group [10], and the medicinal chemistry and mechanism on anti-malaria efficacy has been extensively studied. [11]

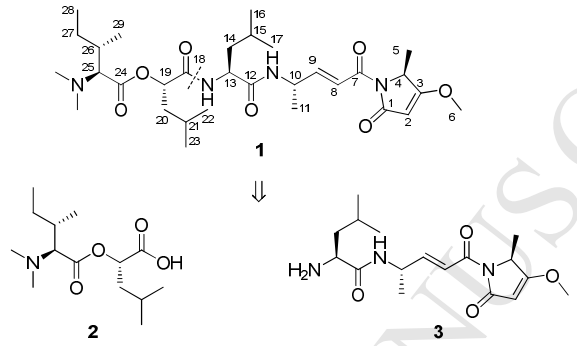
Symplostatin 4 has also been demonstrated to exert moderate activities against Hela and HT-29 cell lines (with IC_{50} of 12 and 53 uM, respectively). [12] However, there have no researching reports concerning its structure−activity relationships (SARs) and mechanistic studies. Structurally, Symplostatin 4 possesses a unique conjugated unsaturated amide system, which might be the key pharmacophore. It is hypothesized that this molecule is a covalent, irreversible inhibitor, as a Michael acceptor, *via* nucleophilic attack by the sulfhydryl side chain of the active site cysteine. Additionally, Symplostatin 4 also possesses an unusual 4(S)-amino-2-(E)-pentenoyl moiety and analiphatic depsipeptide chain. Based on these unique structural features, we envisaged that preparation of Symplostatin 4 and its analogues *via* our optimized synthetic route. It was proposed that the structural modification might provide important alternation on anti-cancer activity and stability of these drug molecules.

RESULTS AND DISCUSSION

Chemistry

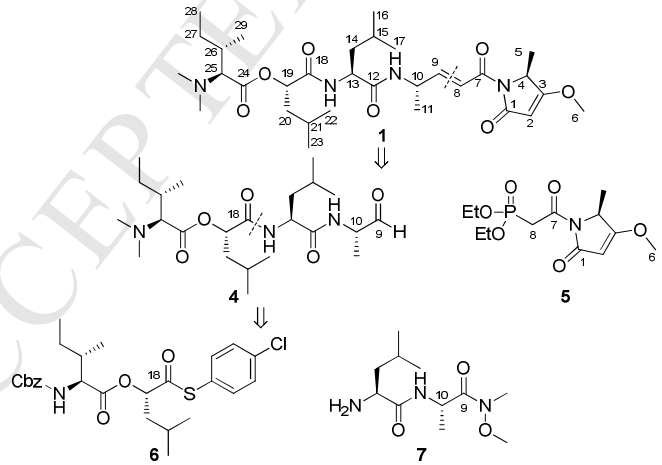
Retrosynthetic analysis of Symplostatin 4

Previously, there were three synthetic reports on the total synthesis of Symplostatin 4. [9, 10, 13] Generally, all of the synthetic strategies were the same, which was disconnected from C18-N amide bond (Scheme 1). The final amide coupling step yielded a messy mixture. The natural molecule **1** could only be purified by preparation HPLC. These synthetic route could not easily generate pure Symplostatin 4 and its analogues in a useful amount, which might greatly impede the anti-cancer medicinal chemistry study on this Symplostatin 4 Scaffold.



Scheme 1. Previous Retrosynthetic Analysis

To this end, an alternative method was employed to efficiently prepare Symplostatin 4. Our retrosynthetic analysis was straightforward, as shown in Scheme 2. Initial disconnection of the conjugated olefin from C8-C9, gave rise to two fragments: the aldehyde **4** and the phosphonate ester **5**. According the modified method, further disconnection through C18-N amide bond led to sulfite ester **6** and amine **7**.

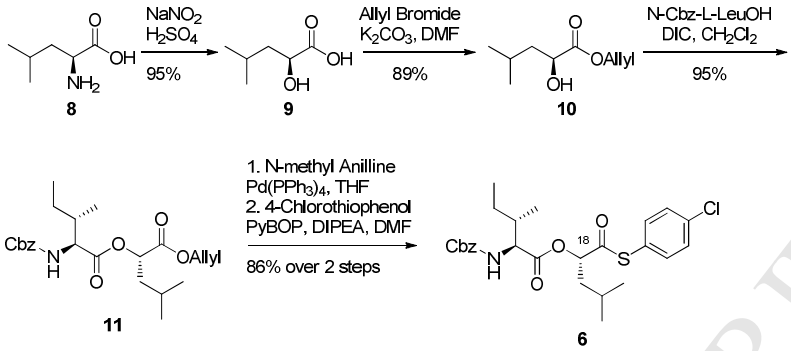


Scheme 2. Alternative Retrosynthetic Analysis

Total synthesis of Symplostatin 4

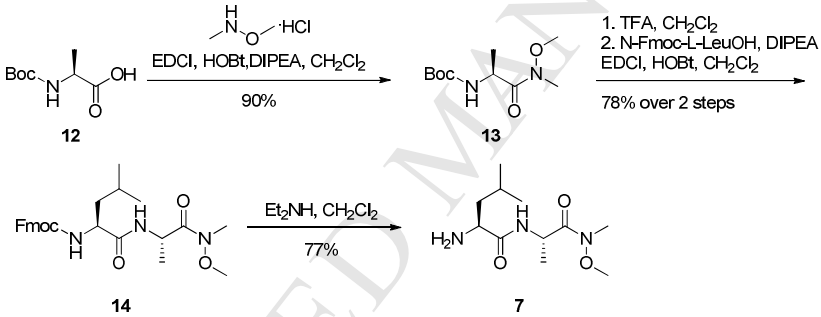
The synthesis of the required fragment **6** started from L-Leu-OH (shown in Scheme 3). Treatment of L-Leu-OH with $NaNO_2$ in acidic aqueous solution produced alcohol **9** in 95% yield. Allyl bromide was employed to protect the carboxylic acid. The resulting intermediate **10** was esterified on the hindered alcohol with N-Cbz-L-Leu-OH under our previous optimized conditions

delivered ester **11**, [14] without any epimerization observed. Cleavage of allyl ester in the presence of Pd(0) catalyst was followed by further coupling with 4-Chlorothiophenol final yield the key fragment **6** in 86% yield over 2 steps.



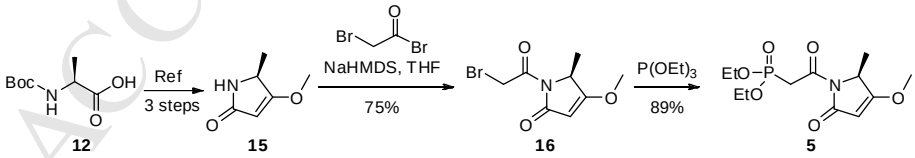
Scheme 3. Synthesis of the Fragment **6**

The synthesis of another key fragment **7** was shown in Scheme 4. A weinreb amide **13** was prepared from the easily available starting material N-Boc-L-Ala-OH. Removal of Boc group by dissolving in TFA solution and coupling with N-Fmoc-L-Leu-OH obtained a dipeptide **14** in 78% overall yield. Deprotection of Fmoc gave rise to compound **7**.



Scheme 4. Synthesis of the Fragment **7**

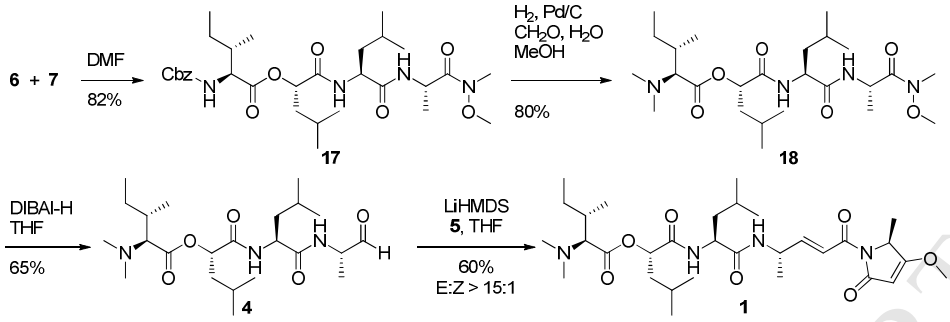
Following preparation of both of the amino skeletons, HWE component **5** was synthesized from known compound **15** in 2 steps (shown in Scheme 5). [15] The compound **15** was pretreated with NaHMDS and was then reacted with 2-bromoacetyl bromide. The resulting **16** heated in neat $P(OEt)_3$ furnished the last fragment **5**.



Scheme 5. Synthesis of the Fragment **5**

With all of the coupling precursors in hands, the depsipeptide chain was constructed in 82% yield by simply mixing intermediate **6** and **7** together in polar solvent. Cbz protecting group was then cleaved and simultaneously di-methylated on the deprotected N-terminal in one-pot reaction under hydrogenation conditions. The weinreb amide was then reduced by DIBAl-H. To our delight, the terminal weinreb amide was reduced without cleavage of the ester bond, even using excess reductant. The resulting aldehyde **4** was connected with compound **5** by HWE

reaction to finally furnish the natural product.



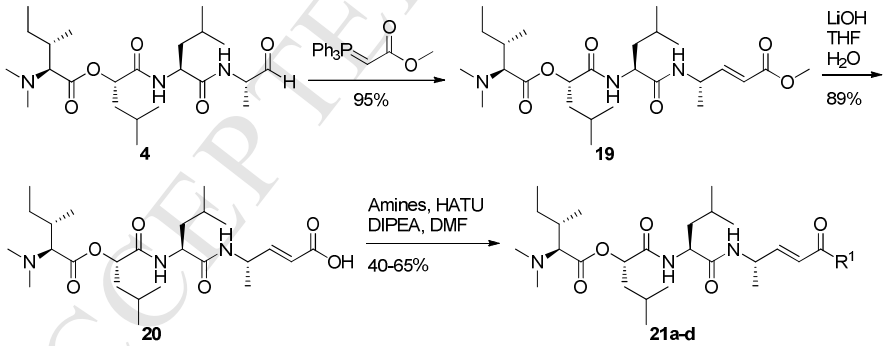
Scheme 6. Fragment Coupling and Total Synthesis of Natural Product

Of note, the final step could be easily purified by flash chromatography on silica gel to obtain the pure Symplostatin 4. More importantly, this synthesis could be achieved in gram-scale to obtain the useful amount of material for medicinal research. By contrast, previously synthetic approaches could only yield several milligrams of pure compound using preparation-HPLC.

Preparation of Symplostatin 4 analogues

Following the success of efficient access to natural product Symplostatin 4, we next investigated the essential anti-cancer pharmacophore, followed by establishment of the preliminary SARs of this natural molecule.

The unusual 4-(S)-amino-2-(E)-pentenoyl moiety (C1-C6 ring fragment) was taken place by some easier amide and ester from the synthetic aldehyde **4** (shown in Scheme 7 and Table 1). Wittig reaction brought conjugated ester **19** in 95% yield. Methyl ester bond was hydrolysis delivered carboxylic acid **20**. Amide coupling using HATU generated the analogues **21a**-**21c**.

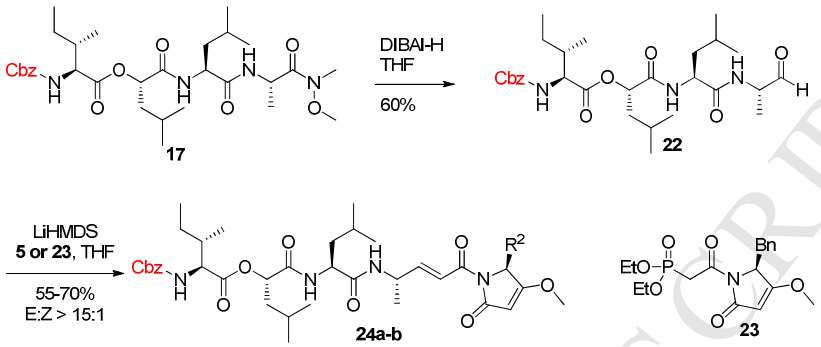


Scheme 7. Replacement of the C1-C6 Fragment

Table 1. Structure of Analogues **21a-c**

Compounds	R^1	Yield
21a		65%
21b		52%
21c		44%

In addition, Cbz functionalized Symplostatin 4 was also synthesized through the same synthetic strategy (Scheme 8 and Table 2). Weinreb amide **17** was reduced by DIBAl-H to generate aldehyde **22**. HWE reaction connected aldehyde **22** with two phosphate ester **5** and **23** to furnish the other two analogues of Symplostatin 4, **24a** and **24b**, with 55% and 70% yield, respectively.

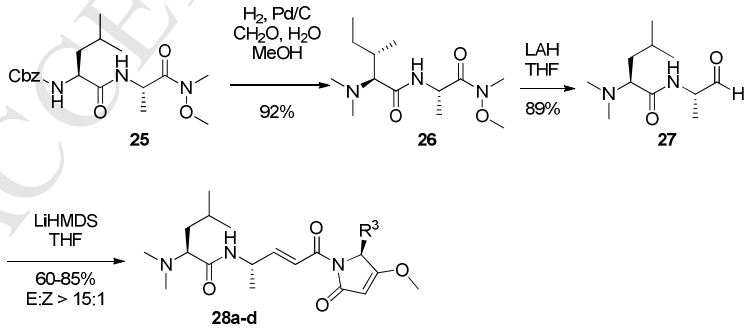


Scheme 8. Synthesis of Symplostatin 4 Analogues

Table 2. Structure of Analogues **24a** and **24b**

Compounds	R^2	Yield
24a	Me	55%
24b	Bn	70%
24c	*i*-Pr	65%

Analogues of this shorter peptide chain was also synthesized to investigate its effect on activity (Scheme 9 and Table 3). The dipeptide **25** was reduced and di-methylated under hydrogenation conditions. The resulting intermediate **26** was converted into aldehyde **27** by LAH reaction, in high yield. HWE reaction connected the aldehyde **27** with different various phosphate esters, which easily derived from amino acids [x]. Four analogues, including **28a**-**28c,** were produced using the same combinatory step.



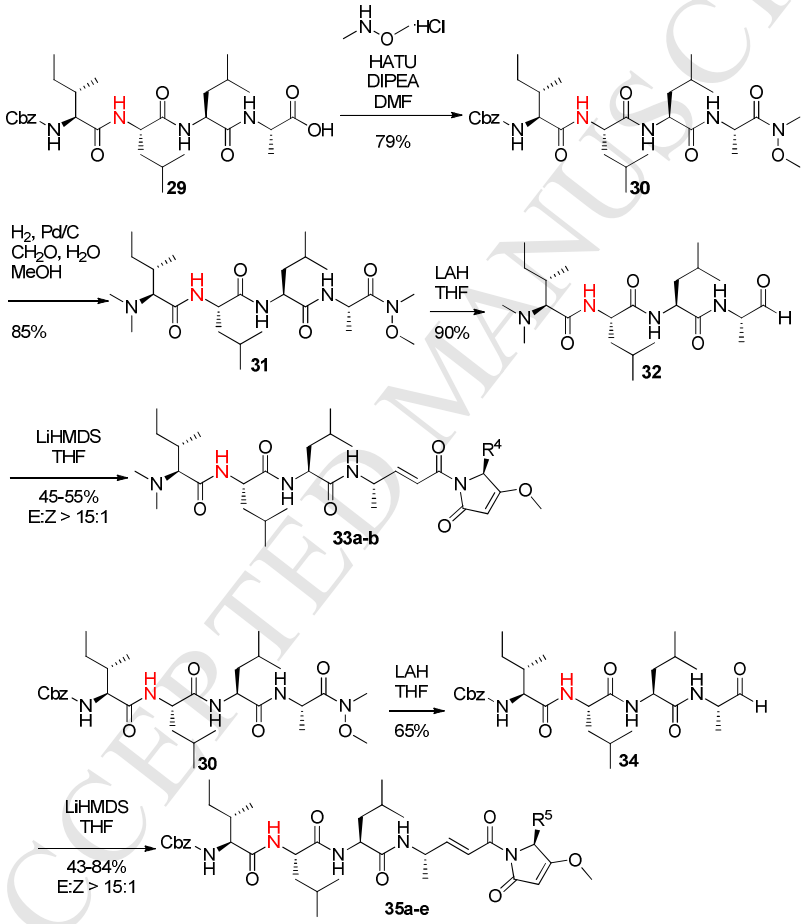
Scheme 9. Synthesis of Symplostatin 4 Analogues

Table 3. Structure of Analogues **28a-c**

Compounds	R^3	Yield
28a	Me	70%
28b	(S)-Bn	85%

28c	(R)-Bn	82%
28d	*i*-Pr	60%

The synthesis of the depsipeptide chain (C9-C31) was 6 linear steps, which was still a rate-limited project. To simplify it, we synthesized the peptide **29** in gram-scale efficiently, instead of the depsipeptide fragment, by employing the solid-phase synthesis (Scheme 10, Table 4 and Table 5). Weinreb amide **30** was obtained from crude peptide **29** by using excess N-methoxy-methylamine hydrochloride. Hydrogenation followed by LAH reduction gave rise to aldehyde analogue **32** in high yield. The same HWE strategy was used to prepare the peptide analogues **33a** and **33b**. Cbz protected compounds **30** was directly reduced by LAH to give N-Cbz-aldehyde intermediate **34**, which was then converted into analogues **35a**-**35e** in the subsequent HWE steps.



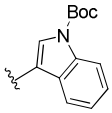
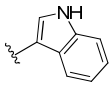
Scheme 10. Synthesis the Symplostatin 4 Analogues

Table 4. Structure of Analogues **33a-b**

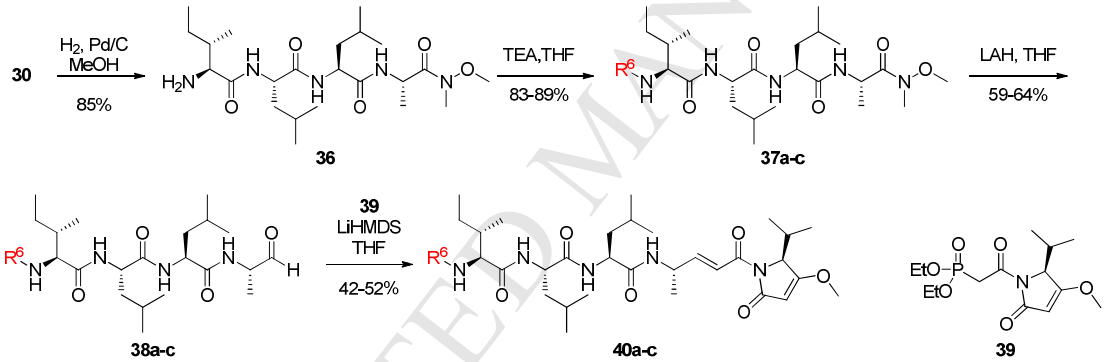
Compounds	R^4	Yield
33a	Me	45%
33b	*i*-Pr	55%

Table 5. Structure of Analogues **35a-e**

Compounds	R^5	Yield

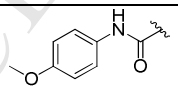
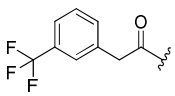
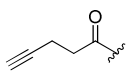
35a	Me	72%
35b	*i*-Pr	43%
35c		79%
35d		82%
35e	(S)-Bn	84%

Following the success of solid-phase synthesis to construct this peptide chain, N-functionalized groups were replaced for the biological evaluation. As shown in Scheme 11 (Table 6), the carbamate group Cbz of common intermediate **30** could be removed under catalytic hydrogenation conditions to obtain the terminal amine **36**. Amine **36** reacted with isocyanate to form a carbamide **37a** with 89% yield. Simultaneously, this amine coupled with carboxylic acids to furnish amides **37b** and **37c** with 83% and 86% yield, respectively. The next two steps were the same as the previously described procedures. Reduction of compound **37a-c** followed by HWE reaction finally obtained Symplostatin 4 analogues **40a-c** in moderate yield.



Scheme 11. Synthesis the N-functionalized Peptides Symplostatin 4 Analogues

Table 6. Structure of Analogues **40a-c**

Compounds	R^6	Step-2 Yield	Step-3 Yield	Step-4 Yield
40a		89%	64%	52%
40b		83%	59%	47%
40c		86%	68%	42%

***In Vitro* Anti-proliferative Activity.**

The growth inhibitory potency of synthesized Symplostatin 4 derivatives was evaluated in three breast cancer cells, two melanoma cells, one pancreatic cancer cell, and one lung cancer cell, using MTT assays as described in the *in vitro* screening protocol. The Symplostatin 4 was included as a comparison (Table 7). The ability of these new analogues to inhibit the growth of

cancer cells was summarized in Table 6. Specifically, the Symplostatin 4 (**1**) exhibited moderate potency against the tested cell lines (IC_{50} was from 31.2 to 45.6 μM), while the compounds **21a-c** and **19** had no inhibitory activity on these cell lines (IC50 > 100 μM), indicating that the unusual 5-member ring moiety (4-amino-2-(E)-pentenoyl) was essential pharmacophore structure. N-Cbz modified analogue **24a** exerted a 2-14 fold more potent antitumor activity than Symplostatin 4 against all examined cell lines. Especially, compound **24a** exhibited remarkable growth inhibition against both A375 and B16F10 cells, with the IC_{50} value of 5.49 and 4.23 μM, respectively, which were 8-fold and 14-fold more potent than Symplostatin 4. However, when Bn group took the place of Me at C-5, the structural modification would tune the anti-proliferative activity of the corresponding analogue **24b**. The analogues **28a-c**, with two amino acid moiety shorterpeptide chain, were subsequently evaluated for anti-proliferative activities on these cell lines. As a result, the compound **28b** with isopropyl substitution at C-5 was found to harbor similar anticancer activity as **23a**. Analogue **28a** with Me group at C-5 led to slightly decreased antitumor activity. While the C-5 substitutions were changed into (R)-Bn (**28b**) or (S)-Bn (**28c**), the growth inhibition effect was almost abolished. Analogue **33a**, which was a peptide analogue of natural product, exhibited increased anti-proliferative activity of cancer cell lines. (4 fold more potent against two melanoma cells, and 2-fold against PANC-1). In comparison with **33a**, peptide analogue **33b** with Isopropyl at C-5 exhibited similar activity as Symplostatin 4. Compounds **35a** and **35b**, which were N-Cbz modified peptide analogues, were found to significantly increase anti-proliferation against breast cancer cells, melanoma cells and pancreatic cancer cells, with IC50 values varying from the low micromolar to submicromolar range. Especially, the compound **35b** was the most potent one. Compared with Symplostatin 4, the IC_{50} values of analogue **35b** were 2.54 μM against MCF-7 (18-fold), 0.922 μM against MDA-MB-231 (38-fold), 0.946 μM against 4T1 (36-fold), 0.890 μM against A375 (51-fold), 0.547 μM against B16F10 (109-fold), 0.787 μM against PANC-1 (39-fold) and 15.1 μM against A549 (2-fold). Alternatively, the synthetic analogues with alternative functional groups on N-terniaml of **35b**, which were urea analogue **40a**, amide analogue **40b** and **40c**, led to dramatically decreased anti-tumor activity against all tested cancer cell lines. Therefore, the compound **35b** was selected as the leading compound for further investigation.

Table 7. Effects of Symplostatin 4 Analogues on Proliferation of Cancer Cell Lines

	IC_{50} (μM) [a]						
	Breast Cancer			melanoma		pancreatic cancer	Lung cancer
Compounds	MCF-7	MDA-MB-231	4T1	A375	B16F10	PANC-1	A549
1	45.5	34.9	34.6	45.6	59.7	31.2	40.4
19	>100[b]	>100	>100	>100	>100	>100	>100
21a	>100	>100	>100	>100	>100	38.3	>100
21b	>100	>100	>100	>100	>100	>100	>100
21c	>100	>100	>100	>100	>100	>100	>100
24a	32.9	15.6	14.96	5.49	4.23	14.0	13.8
24b	>100	>100	79.8	45.9	30.1	59.3	41.4
24c	21.23	12.79	27.89	13.71	24.92	19.59	27.52
28a	75.1	79.7	19.2	11.4	29.2	82.1	30.0
28b	>100	57.7	>100	>100	>100	67	>100

28c	>100	94.8	>100	>100	>100	86.9	>100
28d	18.6	89.45	>100	7.561	30.47	12.76	>100
33a	38.9	38.2	16.5	11.2	15.1	15.7	50.5
33b	>100	98.3	27.6	35.7	61.6	36.0	52.5
35a	4.66	1.96	1.92	1.350	0.892	0.787	15.1
35b	2.54	0.922	0.946	0.801	0.522	0.435	4.66
35c	>100	>100	>100	>100	>100	>100	>100
35d	>100	>100	>100	>100	>100	>100	>100
35e	>100	>100	>100	>100	>100	>100	>100
40a	10.1	25.1	35.3	44.2	60.5	>100	20.8
40b	11.2	32.8	40.8	15.2	15.2	11.6	14.6
40c	14.9	22.2	20.1	9.89	7.07	5.21	36.2

[a] All values are the mean of three independent experiments. [b] If a specific compound is given a value >100, it indicates that a specific IC_{50} cannot be calculated from the data points collected, meaning "no effect".

Compound 35b Inhibited Colony Formation of Melanoma Cells.

In consideration of its potent anti-proliferative activities against two melanoma cancer cells (A375 and B16F10), the leading compound **35b** was selected for colony formation assay. Consequently, compound **35b** exerted an inhibitory effect on colony formation in two malignant melanoma cancer cells A375 and B16F10, as shown in Figure 1, which was consistent with the anti-proliferative activity.

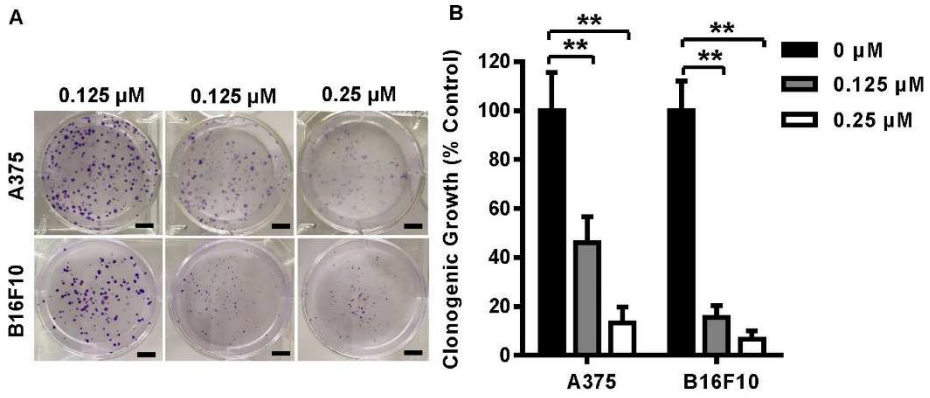


Figure 1. Inhibitory effects of compound **35b** on colony formation of malignant melanoma cancer cells. Colony formation assay was performed to measure the capacity of A375 and B16F10 cells to form colonies. (A) Representative image of long-term growth assay of A375 and B16F10 cells following continuous compound **35b** for 7 days. (B) Quantification of clonogenic growth shown in A. Two way ANOVA; mean ± sd, n = 3. **p<0.01.

Compound 35b Induced Apoptosis of Melanoma Cells.

Based on its promising anti-proliferative effects and potent activities in the colony formation assay, compound **35b** was selected for further mechanistic studies to determine whether the growth inhibition induced by them in human melanoma cells was due to apoptosis. Both A375

and B16F10 cells were treated with vehicle alone as control and also with **35b** at different concentrations for 48 h, following by staining with FITC-Annexin V and propidium iodide (PI). The percentages of apoptotic A375 and B16F10 cells were determined by flow cytometry. As shown in Figure 2, compound **35b** significantly induced apoptosis of A375 and B16F10 cells in a dose-dependent manner. The findings supported that the apoptosis of melanoma cells mediated by this compound contributed to their anti-proliferative effects.

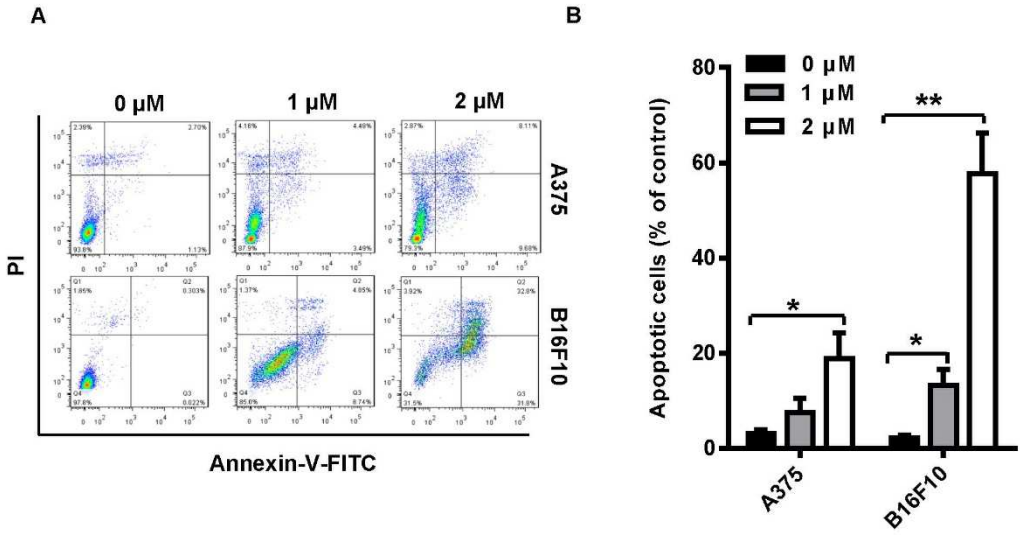


Figure 2. Induction of apoptosis in malignant melanoma cancer cells by compound **35b**. (A) Flow cytometry analysis of apoptotic A375 and B16F10 cells induced by **35b** at different concentrations. (B) Quantitative analysis of apoptosis shown in A. Two-way ANOVA ; mean ± sd, n = 3. * $P < 0.05$; **, $P < 0.01$.

Compound 35b Regulated Apoptotic Related Proteins.

To identify the potential mechanisms of apoptosis induced by compound **35b**, several proteins related to apoptosis (Such as PARP, Caspase 3, Bax and P53) were determined by Western blot (Ref 7-8). As shown in Figure 3, compound **35b** also increased the formation of cleaved PARP and active Caspase-3, two important hallmarks of apoptosis. Consistently, treatment with compound **35b** on A375 and B16F10 cells at different concentrations (0.5-4 μM) for 48 h led to the up-regulation of antiapoptotic protein levels, including Bax and P53 in a dose-dependent manner, which played a vital role in apoptosis. Together, these results indicated that compound **35b** induced apoptosis in melanoma cells.

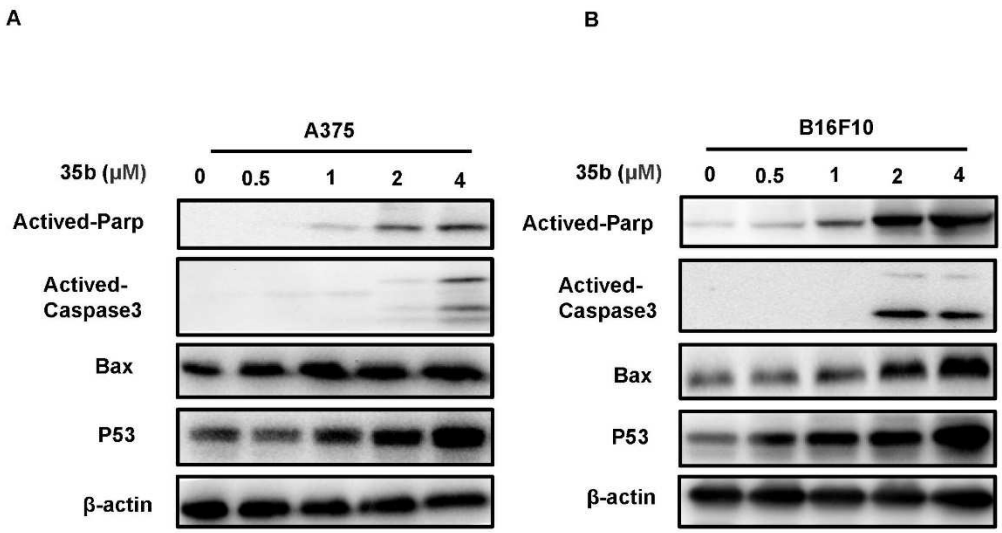


Figure 3. Effect of compound **35b** on apoptosis-related protein levels of malignant melanoma cancer cells. (A) Western blot analysis of biological markers for apoptosis induction by compound **35b** treatment in A375 cells at different concentrations (48 h). (B) Western blot analysis of biological markers for apoptosis induction by compound **35b** treatment in B16F10 cells at different concentrations (48 h).

Compound 35b Inhibited Migration in Melanoma Cells.

To further investigate the motility potential, wound healing assays were performed on A375 and B16F10 cells. As a result, the wound gaps were wider after treatment with compound **35b** on A375 and B16F10 cells (0.25 μM). Meanwhile, Transwell migration assay demonstrated that the number of cells migrating to the lower chamber was also reduced when A375 and B16F10 cells were treated with compound **35b** (0.25 μM) for 24h. Together, the above findings indicated that compound **35b** inhibited the migration ability of A375 and B16F10 cells (Figure 4).

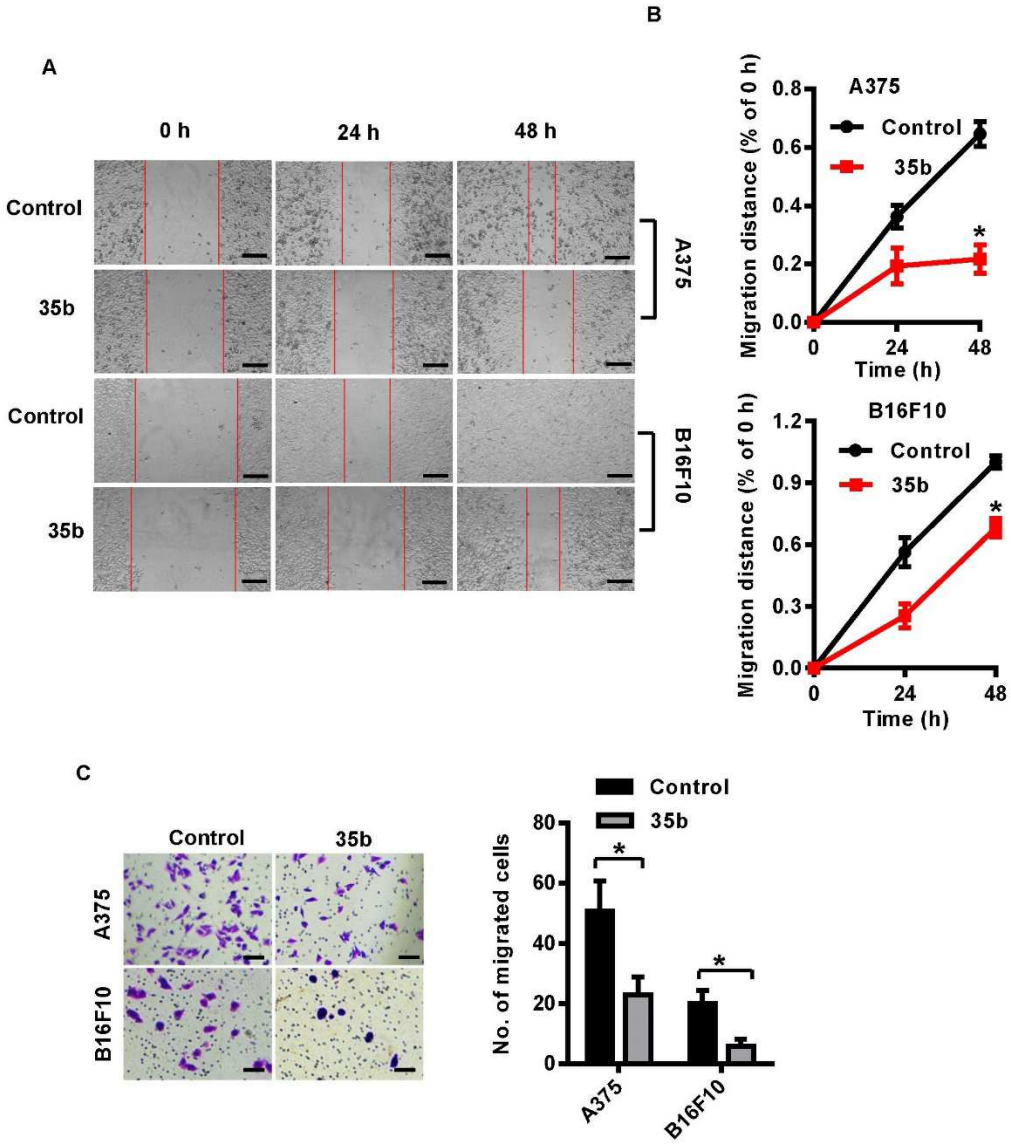


Figure 4. Effect of compound **35b** on migration ability of malignant melanoma cancer cells.(A) A375 and B16F10 cells treated with or without compound **35b** (0.25μM) were subjected to wound-healing assay, representative images were recorded at 0 h, 24 h and 48 h. (B) Quantitative analysis of migration distances shown in A. (C) Cells were seeded into the upper chamber of transwell and incubated with compound **35b** (0.25μM) for 24 h, migration assay were conducted. Representative images of migrated cells were shown (left), quantitative analysis of migrated cells were shown (right). Two-way ANOVA; mean ± sd, n = 3. * $P < 0.05$;

Compound 35b Inhibited Invasion in Melanoma Cells.

Further, transwell invasion assay was used to determine the effect of compound **35b** on cell invasion. As shown in Figure 5, compound **35b** reduced the number of invaded cell through the Matrigel-precoated filter as compared to the control group, indicating that compound **35b** could markedly inhibit the invasion capability of melanoma cells.

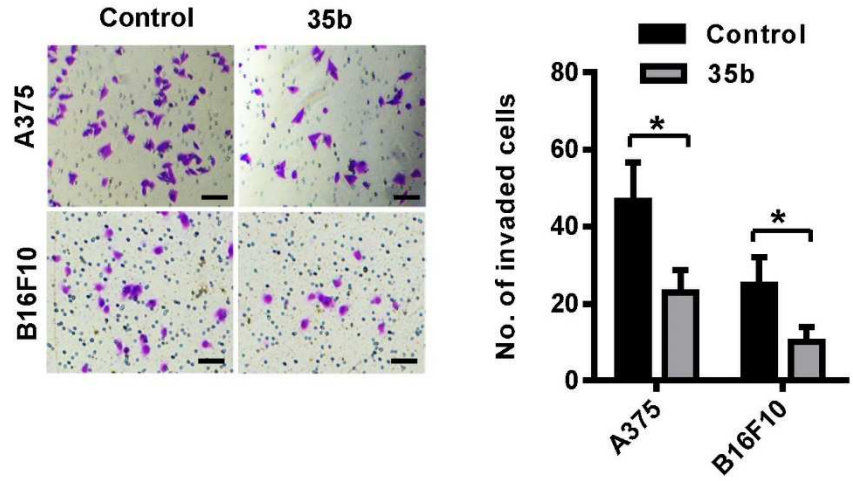


Figure 5. Effect of compound **35b** on invasion ability of malignant melanoma cancer cells.Cells were seeded into the upper chamber of transwell and incubated with compound **35b** (0.25 μM) for 24 h, invasion assay were conducted.Representative images of invaded cells were shown (left), quantitative analysis of invaded cells were shown (right). Two-way ANOVA ; mean ± sd, n = 3. * P < 0.05;

Compound 35b Inhibited the Tumorsphere Formation Ability of Melanoma Cells.

The tumor sphere assays were performed to evaluate whether **35b** inhibited the activity of melanoma CSCs (shown in Figure 6). A375 or B16F10 cells pretreated with or without compound **35b** for 48 h were plated into 24-well ultra-low attachment plates, followed by analysis on the number and size of the tumor spheres after seven days of culture. As a result, compound **35b** administration dramatically decreased the number of tumor spheres, suggesting that compound **35b** administration led to significant inhibition of activity of melanoma CSCs.

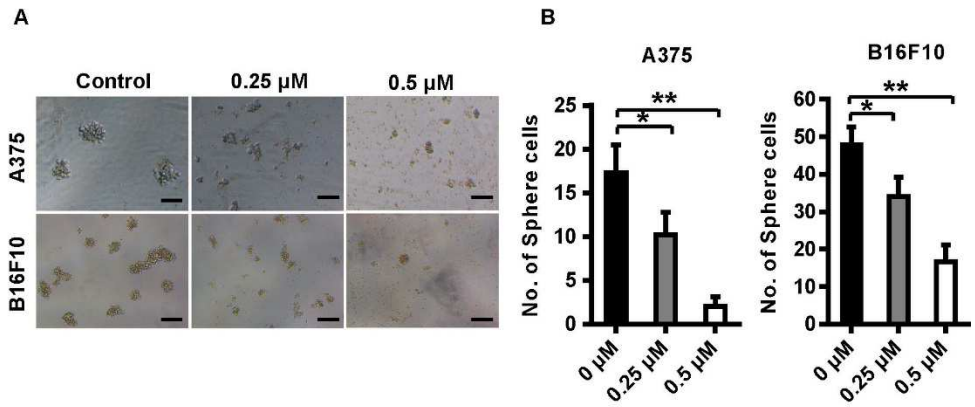


Figure 6. Effect of compound **35b** on tumor spheres formation ability of melanoma cancer cells.(A) The cells were pretreated with or without compound **35b** for 48 h, then collected and plated into 24-well ultra-lowattachment plates. After 1 week of culture, spherical colonies were collected and evaluated. Representative histograms were shown. (B) Quantitative analysis of spherical colonies showed in A. Two-way ANOVA ; mean ± sd, n = 3. * P < 0.05; **, P < 0.01.

Compound 35b Treatment Reduced the Percentage of ALDH$^+$ Cells.

To further investigate the effects of compound **35b** on melanoma CSCs, we assessed whether **35b** decreased the percentage of the ALDH$^+$ cells. A375 and B16F10 cells were exposed to **35b** for 48 h, followed by staining with ALDH and subsequent detection by flow cytometry. [16] The results showed that treatment with **35b** significantly reduced the percentage of ALDH$^+$ cells (Figure. 7), which provided additional evidence for the important role of **35b** in elimination of melanoma CSCs.

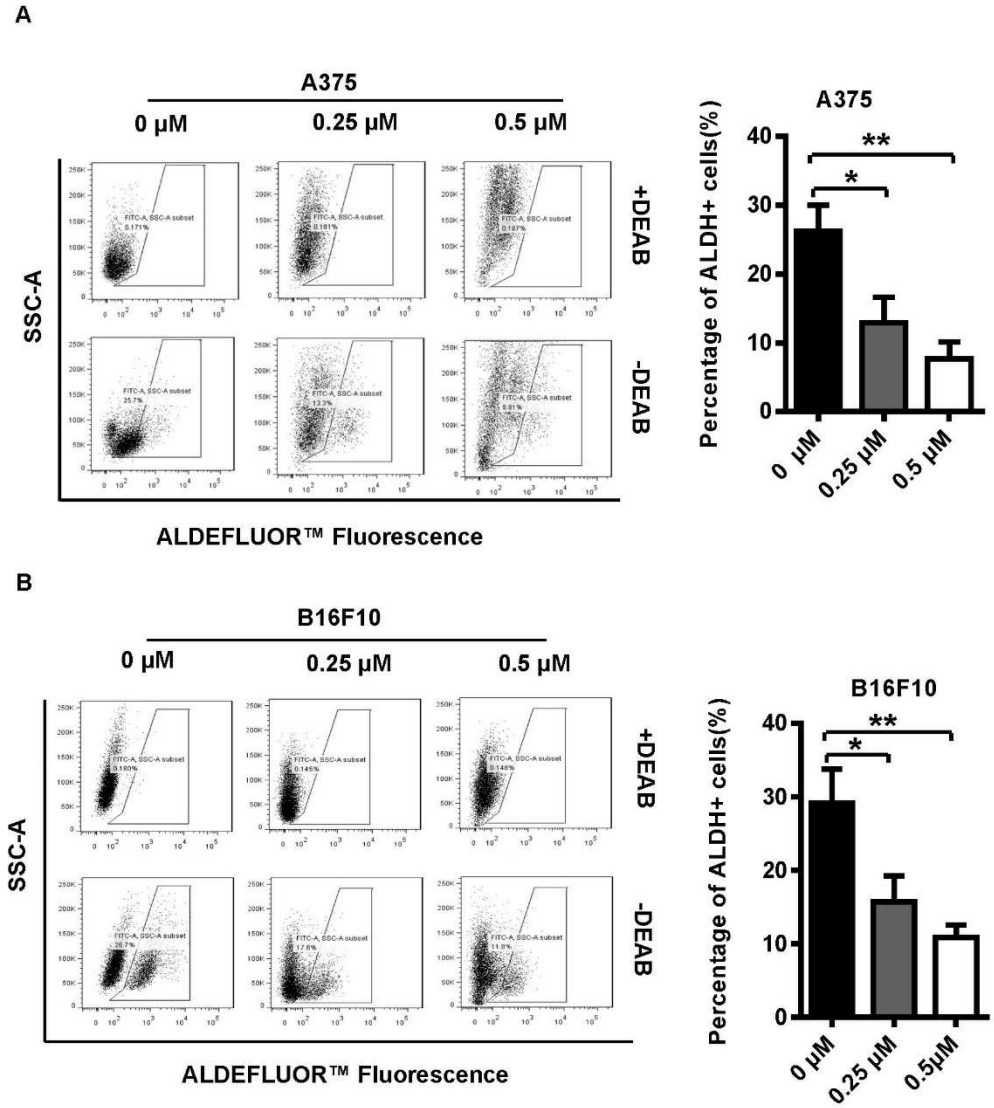


Figure 7. Effect of compound **35b** on ALDH$^+$ subpopulation of malignant melanoma cancer cells.(A) A375 cells were treated with or without compound **35b** for 48 h, the ALDH$^+$ cells were analyzed by flow cytometry. Representative histograms were shown (left), quantitative analysis of ALDH$^+$ cells were showed(right). (B) B16F10 cells were treated with or without compound **35b** for 48 h, the ALDH$^+$ cells were analyzed by flow cytometry. Representative histograms were shown (left),quantitative analysis of ALDH$^+$ cells were showed(right). Two-way ANOVA ; mean ± sd, n = 3. * $P < 0.05$; **, $P < 0.01$.

Compound 35b Treatment Reduced the Percentage of CD133$^+$ Cells.

Convincing evidence from numerous studies indicated that CD133 was an important

biomarker of the CSCs for various cancers, including melanoma. Therefore, to further validate the effects of compound **35b** on melanoma CSCs, we also assessed whether **35b** decreased the percentage of the $CD133^+$ cells. A375 and B16F10 cells were treated with different concentrations of compound **35b** for 48 h, followed by staining with CD133-APC antibody and subsequent detection by flow cytometry. [17] The results showed **35b** could significantly reduce the numbers of $CD133^+$cells (Figure 8), which further confirmed the important role of **35b** in elimination of melanoma CSC

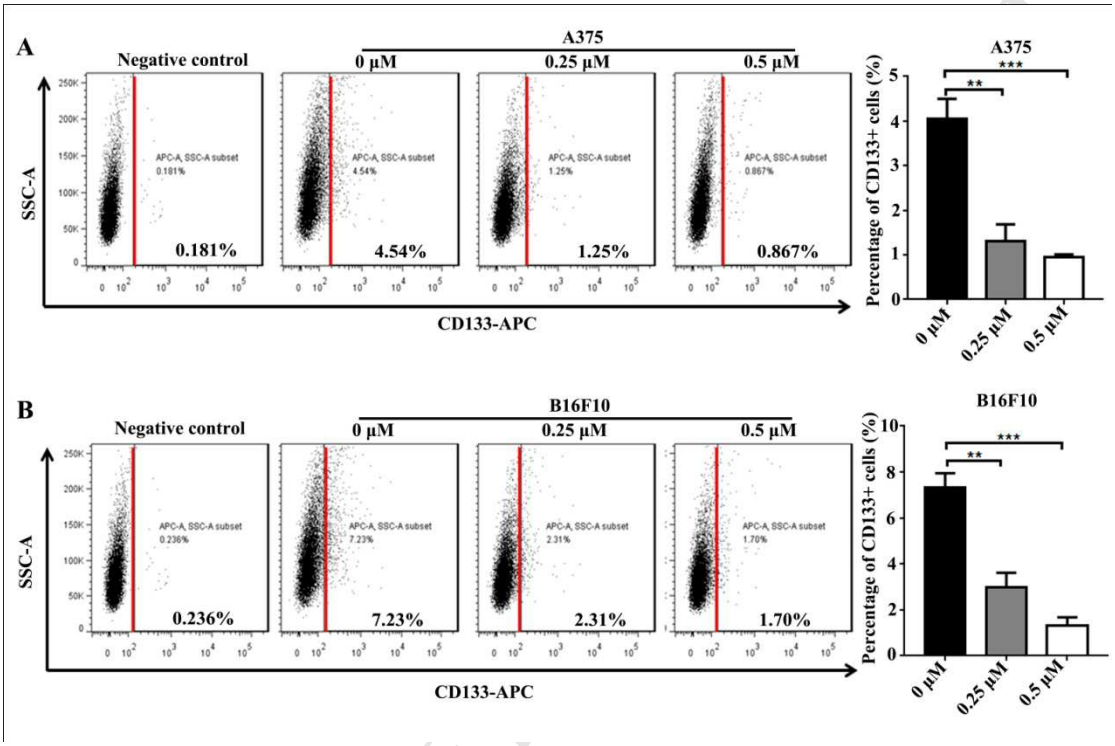


Figure 8. Effect of compound **35b** on $CD133^+$ subpopulation of malignant melanoma cancer cells.(A) A375 cells were treated with or without compound **35b** for 48 h, the $CD133^+$ cells were analyzed by flow cytometry. Representative histograms were shown (left), quantitative analysis of $CD133^+$ cells were showed(right). (B) B16F10 cells were treated with or without compound **35b** for 48 h, the $CD133^+$ cells were analyzed by flow cytometry. Representative histograms were shown (left),quantitative analysis of $CD133^+$ cells were showed(right). Two-way ANOVA ; mean ± sd, n = 3. **, $P < 0.01$, *** $P < 0.001$;

Compound 35b Inhibited crosstalk pathway between STAT3 and Wnt/β-catenin Signaling Pathway in Melanoma Cells.

Further mechanism studies on the anti-CSCs effects of compound **35b** aimed to determine the possible signaling pathway, which might be blocked following compound **35b** administration. Firstly, luciferase reporter assays indicated the compound **35b** could efficiently inhibit the activity of TCF/LEF and markedly block the Wnt signaling pathway (shown in Figure 9A). Additionally, Western blot revealed that compound **35b** decreased the phosphorylation level of STAT3, protein level of β-catenin and its downstream targets c-Myc in dose-dependent manners (Figure. 9B and C). Collectively, these data suggested that compound **35b** effectively eliminated CSCs of melanoma cells by blocking the crosstalk between STAT3 and Wnt/β-catenin signaling pathways.

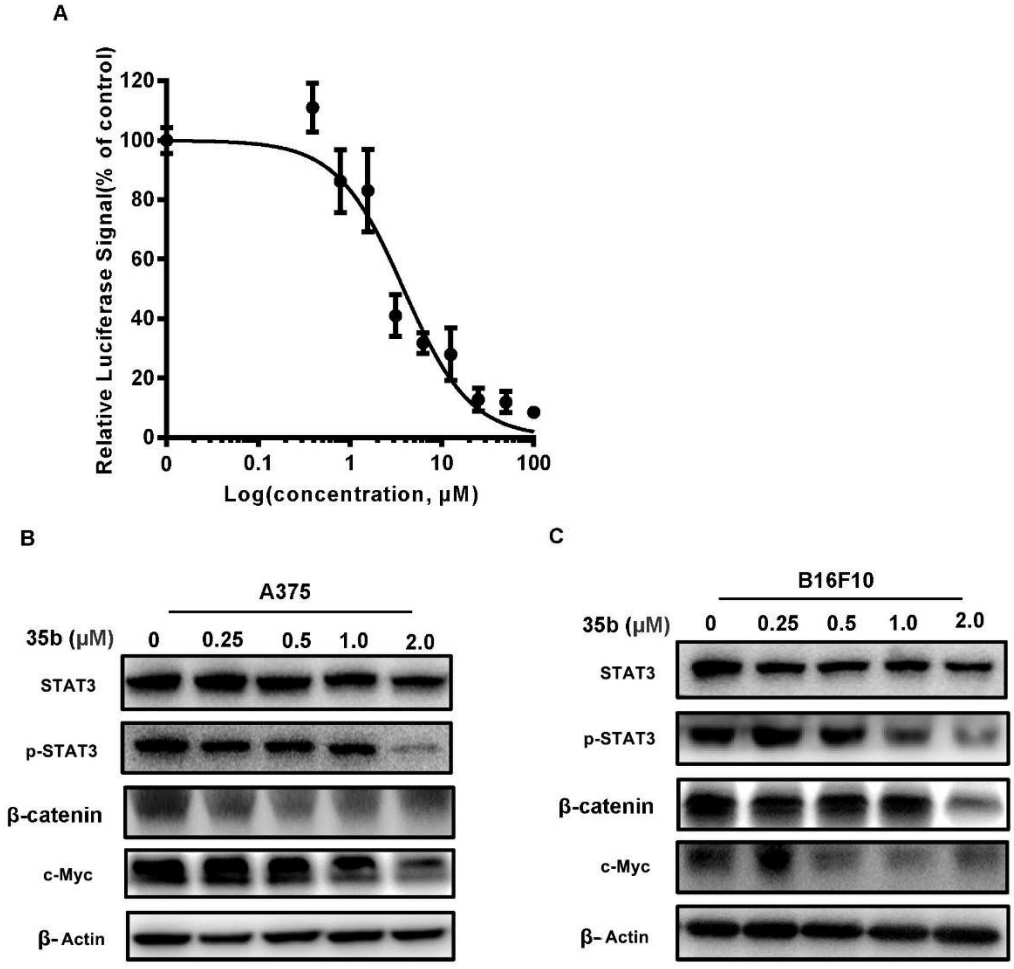


Figure 9. Comound **35b** inhibited STAT3 and Wnt/β-catenin signaling pathway in malignant melanoma cancer cells. (A) 293T cells transfected with TCF/LEF reporter plasmid were treated with compound **35b** at different concentrations (48 h). Cells were harvested for luciferase assay, luciferase activity was normalized to Renilla luciferase activity.(B) Western blot analysis of STAT3, p-STAT3, β-catenin and c-Myc in the A375 cells treated by compound **35b** at different concentrations (48 h).(C)Western blot analysis of STAT3, p-STAT3, β-catenin and c-Myc in the B16F10 cells treated by compound **35b** at different concentrations (48 h).

Compound 35b Suppressed B16F10 Tumor Growth *in vivo*.

The leading compound **35b** was further evaluated for its anticancer activity *in vivo* for its suppression of B16F10 tumor growth. Consequently, tumor sizes were significantly suppressed in mice treated with compound **35b** in comparison with that in control group (Figure 10). Moreover, there was no obvious difference of body weight in two groups. These results indicated that compound **35b** could distinctly inhibit B16F10 tumor growth *in vivo.*

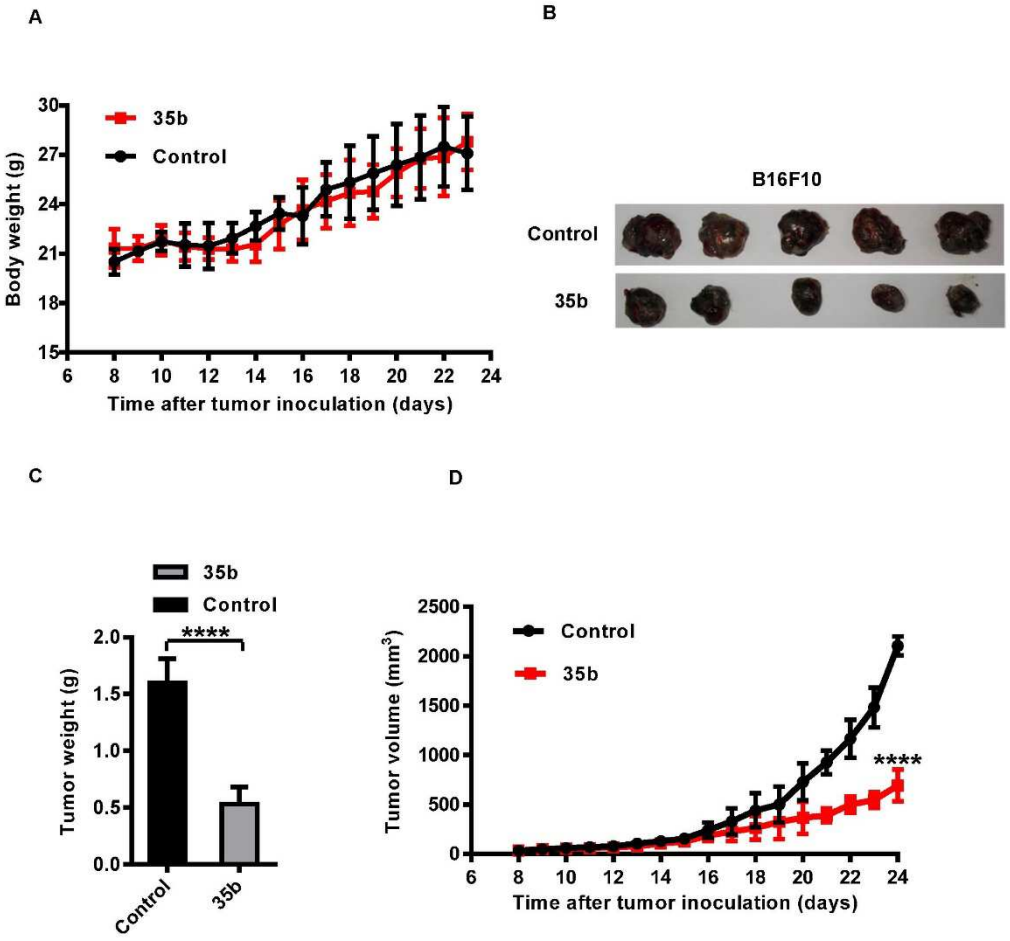


Figure 10. Effect of compound **35b** on B16F10 tumor growth delay and regression in vivo.C57BL/6J mice were subcutaneously injected with B16F10 cells. (A) Body weights of animals. (B) The representative images of tumors dissected from vehicle-and compound **35b**-treated mice were shown. (C) Tumors were weighted on day 24. (D) Changes in the tumor volume of B16F10 xenografts. Two-way ANOVA ; mean ± sd, n = 5.****, P < 0.0001.

CONCLUSIONS

In this report, natural product Symplostatin 4 was totally synthesized through an alternative and efficient manner. The whole synthesis provided gram-scale natural product Symplostatin 4 in 8 linear steps with 6% overall yield. And the purification process could be more straightforward than the previously reported synthetic route. Based on these successful achievement, 22 synthetic analogues from Symplostatin 4 scaffold were prepared and evaluated by anti-tumor assays. Among them, the compound **35b** was demonstrated as a leading compound with great suppression effect of tumor growth both *in vitro* and *in vivo*. Furthermore, the compound **35b** could significantly reduce the number of the tumor spheres and decrease the percentage of $ALDH^+$ melanoma cells, indicating that this leading compound **35b** possessed the potent activity against CSCs. Further mechanism study illustrated that compound **35b** could ablate the CSCs of melanoma through blocking the crosstalk between STAT3 and Wnt/β-catenin signaling pathways. In summary, the leading compound **35b** was a promising anti-CSCs agent that merited further investigation.

Experimental section

*(S)-2-Hydroxy-4-methylpentanoic acid (**9**).* A three-neck flask was charged with L-Leu-OH (10.0 g, 76.2 mmol), H_2O (50 mL) and concentrated sulfuric acid (23.3 g, 91.4 mmol). The resulting solution was cooled to 0 °C. A solution of $NaNO_2$ (7.89 g, 0.114 mol, in 50 mL H_2O) was then added dropwise. The reaction mixture was stirred at room temperature overnight. The mixture was extracted with ethyl acetate (3 × 100 mL). The combined organic layers were washed with brine (100 mL), dried over anhydrous $NaSO_4$ and filtered. The solvent was concentrated *in vacuo* to afford acid **9** as a pale yellow oil, which could be used directly without any further purification (9.50 g, 95%). $[\alpha]_D^{20}$= –21, (c = 1.0, MeOH). v_{max} (KBr): 3424, 2960, 2873, 2629, 1726, 1650, 1432, 1389, 1370 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 4.29 (dd, *J* = 8.1, 5.3 Hz, 1H), 1.91 (dp, *J* = 13.4, 6.7 Hz, 1H), 1.63 (ddd, *J* = 8.1, 5.4, 1.9 Hz, 2H), 0.97 (d, *J* = 6.6 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 180.7, 69.0, 43.4, 24.6, 23.3, 21.5. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_6H_{12}O_3Na^+$, 155.0679; found, 155.0677.

*Allyl-(S)-2-hydroxy-4-methylpentanoate (**10**).* A round-bottom flask was charged with the acid **9** (5.00 g, 37.8 mmol), K_2CO_3 (10.5 g, 75.7 mmol) and dry DMF (70 mL). Allyl bromide (9.15 g, 75.7 mmol) was added and the resulting suspension was stirred overnight in a nitrogen atmosphere. After the substrate was completely consumed (monitored by TLCTLC analysis), the reaction mixture waswashed with H_2O (1000 mL), and extracted by ethyl acetate (3 × 100 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (petroleum ether: ethyl acetate = 20:1 to 5:1) to afford desired compound **10** as a pale yellow oil (5.81 g, 89%). $[\alpha]_D^{20}$ = –13.6, (c = 1.0, MeOH). v_{max} (KBr): 3474, 3087, 2958, 2872, 2377, 1737, 1649, 1469, 1386, 1368, 1270 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 5.92 (ddt, *J* = 17.1, 10.4, 5.8 Hz, 1H), 5.40 – 5.22 (m, 2H), 4.72 – 4.61 (m, 2H), 4.22 (ddd, *J* = 8.3, 5.9, 4.9 Hz, 1H), 2.67 (d, *J* = 6.0 Hz, 1H), 1.90 (ddt, *J* = 14.5, 13.2, 6.7 Hz, 1H), 1.61 – 1.52 (m, 2H), 0.95 (dd, *J* = 6.7, 4.2 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.7, 131.6, 119.1, 69.2, 66.2, 43.6, 24.5, 23.3, 21.7. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_9H_{16}O_3Na^+$, 195.0992; found, 195.0991.

*Allyl (R)-2-((((benzyloxy) carbonyl)-L-isoleucyl) oxy)-4-methylpentanoate (**11**).* A round-bottom flask was charged with N-Cbz-L-Ile-OH(13.0 g, 49.0 mmol), compound **10** (2.80 g, 16.3 mmol) , DIC (6.18g, 49.0 mmol), and dichloromethane (55 mL). The resulting solution was cooled to 0 °C. The reaction mixture was stirred for 15 min. DMAP (599 mg, 4.90 mmol) was added and the resulting solution was stirred for further 6 h. The reaction mixture was washed with 1 N hydrochloric acid solution, saturated sodium carbonate solution and brine. The combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (petroleum ether: ethyl acetate= 30: 1 to 20:1) to afford desired compound **11** as a pale yellow oil (6.50 g, 95%). $[\alpha]_D^{20}$ = –25.4, (c = 1.0, MeOH). v_{max}(KBr): 3383, 2963, 2935, 2875, 1711, 1574, 1514, 1477, 1389, 1338, 1231, 1154, 1088, 980, 855, 748, 697 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 7.26 (d, *J* = 4.4 Hz, 5H), 5.85 – 5.72 (m, 1H), 5.27 – 5.10 (m, 3H), δ 5.04 – 4.95 (m, 3H), 4.52 (d, *J* = 5.7 Hz, 2H), 4.33 (dd, *J* = 9.0, 4.3 Hz,

1H), 1.88 (dt, *J* = 10.9, 5.5 Hz, 1H), 1.70 (ddt, *J* = 23.9, 12.7, 5.6 Hz, 2H), 1.57 (td, *J* = 8.2, 4.1 Hz, 1H), 1.51 – 1.39 (m, 1H), 1.21 – 1.06 (m, 1H), 0.96 – 0.74 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) *δ* 171.8, 170.0, 156.2, 136.4, 131.6, 128.7, 128.3, 128.2, 119.1, 71.9, 67.1, 66.0, 58.3, 39.9, 38.1, 24.7, 24.6, 23.1, 21.7, 15.4, 11.8. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{23}H_{33}NO_6Na^+$, 442.2200; found, 442.2199.

*(S)-1-((4-Chlorophenyl) thio)-4-methyl-1-oxopentan-2-yl ((benzyloxy) carbonyl)-L-isoleucinate (**6**).* A round-bottom flask was charged with compound **11** (4.0 g, 9.4 mmol), Morpholine (1.6 g，19 mmol), $Pd(Ph_3)_4$ (0.6 g, 0.5 mmol) and THF (10 mL). The resulting suspension was stirred for 7h. After the substrate was completely consumed (monitored by TLC analysis), The reaction mixture was washed with 1 N hydrochloric acid solution, and extracted by ethyl acetate (3 × 20 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo* to afford an intermediate (3.6 g). To solution of the intermediate in DMF (20 mL) without any further purification, followed by PyBOP (3.2 g, 6.1 mmol) and DIPEA (0.8 g, 6.1 mmol). The resulting solution was stirred overnight. The reaction mixture was washed with 1 N hydrochloric acid solution, and extracted by ethyl acetate (3 × 20 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo.* The residue was purified by flash column chromatography on silica gel (petroleum ether: ethyl acetate= 10:1) to afford fragment **6** as a pale yellow oil (2.6g, 86% for two steps). $[\alpha]_D^{20}$ = – 62, (c = 1.0, MeOH). ν_{max}(KBr) = 3474, 2958, 2872, 2377, 1737, 1649, 1469, 1368, 1205, 1141, 1088, 932, 851, 748 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.34 (ddd, *J* = 16.0, 14.1, 8.5 Hz, 10H), 5.40 (dd, *J* = 9.4, 3.6 Hz, 1H), 5.33 (d, *J* = 9.2 Hz, 1H), 5.13 (s, 2H), 4.50 (dd, *J* = 9.2, 4.4 Hz, 1H), 2.11 (dd, *J* = 10.0, 6.5 Hz, 1H), 1.94 – 1.66 (m, 3H), 1.54 (ddd, *J* = 13.2, 7.4, 4.0 Hz, 1H), 1.27 (d, *J* = 2.9 Hz, 1H), 1.04 (d, *J* = 6.8 Hz, 3H), 1.01 – 0.91 (m, 10H). ^{13}C NMR (100 MHz, $CDCl_3$) *δ* 196.7, 171.33, 156.7, 136.8, 136.2, 129.7, 128.6, 128.3, 128.2, 124.6, 77.9, 67.2, 58.6, 40.9, 37.5, 24.5, 24.4, 23.1, 21.6, 15.7, 11.6. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{23}H_{33}NO_6Na^+$, 528.1582; found, 528.1580.

*Tert-butyl (S)-(1-(methoxy (methyl) amino)-1-oxopropan-2-yl) carbamate (**13**).* A round-bottom flask was charged with N-Boc-L-Ala-OH (5.0 g, 26 mmol), N,O-dimethylhydroxylamine hydrochloride (3.1 g, 32 mmol), HOBT (4.3 g, 32 mmol), DIPEA (17 g, 0.13 mol), EDCI (6.1 g, 0.32 mol) and dichloromethane(100 mL). The resulting suspension was cooled to 0 °C and was stirred overnight. The reaction mixture was washed with 1 N hydrochloric acid solution, saturated sodium carbonate solution, and extracted by ethyl acetate. The combined organic phase were dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo.* The residue was purified by flash column chromatography on silica gel (petroleum ether: ethyl acetate= 5:1 to 3: 1) to afford weinreb amide **13** as a white solid (5.5 g, 90%). $[\alpha]_D^{20}$ = –21.5, (c = 1.0, MeOH). m. p.139 ℃. ν_{max}(KBr):3293, 3049, 3008, 2975, 2823, 1660, 1540, 1456, 1423 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) *δ* 5.25 (d, *J* = 7.4 Hz, 1H), 4.66 (t, *J* = 7.6 Hz, 1H), 3.75 (s, 3H), 3.19 (s, 3H), 1.42 (s, 9H), 1.29 (d, *J* = 6.9 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) *δ* 173.8, 155.3, 79.6, 61.7, 46.6, 32.2, 28.4, 18.8. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{10}H_{20}N_2O_4Na^+$, 255.1315; found, 255.1314.

(9H-fluoren-9-yl)methyl((S)-1-(((S)-1-(methoxy(methyl)amino)-1-oxopropan-2-yl)amino)-4-methyl-

*1-oxopentan-2-yl) carbamate (**14**)*. A three-neck flask was charged with weinreb amide **13** (3.00 g, 11.2 mmol) and dichloromethane (9 mL). The resulting solution was cooled to 0 °C in a nitrogen atmosphere. TFA (17.2 g, 112 mmol) was then added slowly. The reaction mixture was stirred 2 h. The solvent was concentrated *in vacuo* to afford an intermediate. To solution of the intermediate (1.40 g, 6.20 mmol) in dichloromethane (40 mL). N-Fmoc-L-Leu-OH (2.20 g, 6.20 mmol), HOBT (1.00 g, 7.44 mmol), DIPEA (4.00 g, 31.0 mmol) and EDCI (1.40 g, 1.44 mmol) was added under nitrogen atmosphere. The resulting solution was stirred overnight. The reaction mixture was washed with brine and dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (petroleum ether: ethyl acetate= 3:1 to 1: 1) to afford weinreb amide **14** as a white solid (2.25 g, 78% for two steps). $[\alpha]_D^{20}$ = –37.6, (c = 1.0, MeOH). m. p. 92 °C. ν_{max} (KBr)= 3301, 3069, 2957, 2871, 1717, 1642, 1536, 1468, 1450, 1392, 1369, 1323, 1238, 1181, 1042, 759, 740 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.5 Hz, 2H), 7.61 – 7.57 (m, 2H), 7.39 (t, *J* = 7.3 Hz, 2H), 7.30 (t, *J* = 7.3 Hz, 2H), 6.83 (d, *J* = 7.1 Hz, 1H), 5.35 (d, *J* = 8.6 Hz, 1H), 4.92 (t, *J* = 6.7 Hz, 1H), 4.39 (dt, *J* = 17.6, 10.4 Hz, 2H), 4.29 – 4.19 (m, 2H), 3.77 (s, 3H), 3.21 (s, 3H), 1.69 – 1.51 (m, 3H), 1.35 (d, *J* = 6.8 Hz, 3H), 0.94 (d, *J* = 5.7 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.8, 171.8, 156.2, 144.0, 143.8, 141.3, 127.8, 127.1, 125.2, 120.1, 67.0, 61.7, 53.5, 47.2, 45.8, 42.1, 32.3, 24.7, 23.1, 21.9, 18.3. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{26}H_{33}N_3O_5Na^+$,490.2312; found, 490.2311.

*(S)-2-amino-N-((S)-1-(methoxy(methyl)amino)-1-oxopropan-2-yl)-4-methylpentanamide (**7**).* A round-bottom flask was charged with weinreb amide **14** (1.00 g, 2.14 mmol), dichloromethane (30 mL). Diethylamine (15 mL) was added and the resulting solution was stirred for 2 h. The reaction mixture was washed with brine and extracted by ethyl acetate (3 × 30 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo.* The residue was purified by flash column chromatography on silica gel (petroleum ether: ethyl acetate= 4:1 to diethylamine : methanol = 4: 1) to afford desired compound **7** as a white solid (406 mg, 77%). $[\alpha]_D^{20}$ = – 0.1, (c = 1.0, MeOH). m. p. 181 °C. ν_{max}(KBr): 3197, 3086, 2958, 2872, 1687, 1447, 1346, 1151, 1110 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.14 (d, *J* = 7.7 Hz, 1H), 4.83 – 4.64 (m, 1H), 3.73 (s, 3H), 3.15 (dd, *J* = 9.2, 4.9 Hz, 1H), 3.11 (s, 3H), 1.73 (dtd, *J* = 15.0, 8.6, 8.0, 4.7 Hz, 3H), 1.39 (ddd, *J* = 13.5, 8.7, 4.9 Hz, 1H), 1.24 – 1.14 (m, 3H), 0.86 (dd, *J* = 14.5, 6.6 Hz, 6H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 175.3, 61.2, 52.7, 44.3, 44.2, 24.0, 23.3, 21.7, 17.5. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{11}H_{23}N_3O_3Na^+$, 268.1632; found, 268.1632.

*(S)-4-Methoxy-5-methyl-1,5-dihydro-2H-pyrrol-2-one (**15**).* A round-bottom flask was charged with Boc-**15** (4.00 g, 17.6 mmol) and dichloromethane (13 mL). The resulting solution was cooled to 0 °C. TFA (17.2 g, 112 mmol) was then added slowly. The reaction mixture was stirred 1 h. After the substrate was completely consumed (monitored by TLC analysis), toluene was added. The reaction mixture was concentrated *in vacuo.* The residue was purified by flash column chromatography on silica gel (petroleum ether: ethyl acetate= 1:1 to diethylamine : methanol = 4: 1) to afford desired compound **15** as a yellow solid (2.20 g, 97%). $[\alpha]_D^{20}$ = +13, (c = 1.0, MeOH). m. p. 112 °C. ν_{max}(KBr): 3256, 2942, 2881, 2605, 2310, 1680, 1620, 1456, 1379, 1361, 1233, 1207, 1178, 1049, 987, 811 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.88 (brs, 1H), 4.97 (s, 1H), 4.06 (d, *J* = 6.6 Hz, 1H), 3.77 (s, 3H), 1.29 (d, *J* = 6.7 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 179.6, 174.6, 92.9, 58.4, 53.4, 17.8. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_6H_9NO_2Na^+$, 150.0525; found, 150.0525.

*(S)-1-(2-Bromoacetyl)-4-methoxy-5-methyl-1,5-dihydro-2H-pyrrol-2-one (**16**).* A three-neck flask was charged with compound **15** (2.00 g, 15.7 mmol) and dry THF (60 mL). The resulting solution was cooled to –78 °C in a nitrogen atmosphere. NaHMDS (7.90 mL, 15.7 mmol) was added dropwise. The reaction mixture was stirred for 15 min. Bromoacetyl bromide (2.60 g, 13.0 mmol) was then added dropwise over 10 min. The resulting solution was stirred for further 3 h. After the substrate was completely consumed (monitored by TLC analysis), the reaction mixture was quenched with saturated NH_4Cl solution, and extracted by ethyl acetate (3 × 100 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (petroleum ether: ethyl acetate= 5:1 to 1: 1) to afford desired compound **16** as a yellow solid (2.90 g, 75%). $[\alpha]_D^{20}$ = + 84, (c = 1.0, MeOH). m. p. 75 °C. ν_{max}(KBr): 3115, 3043, 2969, 2944, 1721, 1692, 1619, 1454, 1344, 1189, 1094, 956, 812 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 4.95 (s, 1H), 4.51 – 4.42 (m, 2H), 4.37 (d, *J* = 12.8 Hz, 1H), 3.77 (s, 3H), 1.37 (d, *J* = 6.6 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 181.1, 169.3, 165.1, 92.5, 59.0, 56.0, 30.7, 16.6. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_8H_{10}BrNO_3Na^+$, 269.9736; found, 269.9733.

*Aiethyl (S)-(2-(3-methoxy-2-methyl-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-2-oxoethyl) phosphonate (**5**).* A round-bottom flask was charged with compound **16** (1.0 g, 4.0 mmol) and triethyl phosphite (3.3 g, 20 mmol). The resulting solution was stirred overnight in 55 °C. After the substrate was completely consumed (monitored by TLC analysis), the reaction mixture was concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (petroleum ether: ethyl acetate= 2:1 to dichloromethane: methanol=10: 1) to afford phosphonate ester **5** as a pale yellow oil (1.2 g, 89%). $[\alpha]_D^{20}$ = +59.9, (c = 1.0, MeOH). ν_{max}(KBr): 3099, 2985, 2941, 1726, 1682, 1626, 1455, 1382, 1331, 1251, 1170, 1025, 970 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 5.02 (s, 1H), 4.57 (d, *J* = 6.3 Hz, 1H), 4.14 (t, *J* = 6.9 Hz, 4H), 3.93 (dd, *J* = 22.2, 14.2 Hz, 1H), 3.84 (s, 3H), 3.93 (dd, *J* = 22.2, 14.2 Hz, 1H), 1.45 (d, *J* = 6.3 Hz, 3H), 1.29 (t, *J* = 6.9 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 180.8, 168.5, 164.1, 164.0, 92.1, 62.5, 58.9, 55.9, 36.4, 35.1, 16.9, 16.4, 16.3. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{12}H_{20}NO_6PNa^+$, 328.0920; found, 328.0918.

*(S)-1-(((S)-1-(((S)-1-(Methoxy (methyl) amino)-1-oxopropan-2-yl) amino)-4-methyl-1-oxopentan-2-yl) amino)-4-methyl-1-oxopentan-2-yl ((benzyloxy)carbonyl)-L-isoleucinate (**17**).* A round-bottom flask was charged with fragment **6** (1.84 g, 7.50 mmol), compound **7** (4.50 g, 9.00 mmol) and DMF (140 mL). The resulting solution was stirred overnight in room temperature. The reaction mixture was washed with water (200 mL) and extracted by ethyl acetate (3 × 300 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (petroleum ether: ethyl acetate= 1:1) to afford desired compound **17** as a pale yellow oil (3.70 g, 82%). $[\alpha]_D^{20}$ = – 55, (c = 1.0, MeOH). ν_{max}(KBr): 3301, 2961, 2874, 2377, 2310, 1725, 1653, 1533, 1456, 1339, 1046 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 7.39-7.30 (m, 5H), 6.95 (d, *J* = 7.8 Hz, 1H), 6.77 (d, *J* = 7.1 Hz, 1H), 5.25-5.07 (m, 4H), 4.94-4.85 (m, 1H), 4.53-4.44 (m, 1H), 4.38-4.36 (m, 1H), 3.74 (s, 3H), 3.19 (s, 3H), 2.07-1.99 (m, 1H), 1.77-1.54 (m, 6H), 1.47-1.39 (m, 2H), 1.31 (d, *J* = 6.7 Hz, 3H), 1.27-1.22 (m, 1H), 1.00 (d, *J* = 6.4 Hz, 3H), 0.96-0.83 (m, 14H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.7, 171.2, 171.1, 170.1, 157.1, 136.0, 128.8,

128.5, 128.1, 74.1, 67.3, 61.7, 59.0, 51.5, 45.6, 41.0, 40.6, 37.2, 32.2, 25.1, 24.8, 24.7, 23.3, 21.7, 18.2, 16.0, 11.8. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{31}H_{50}N_4O_8Na^+$, 629.3521; found, 629.3520.

*(S)-1-(((S)-1-(((S)-1-(Methoxy(methyl)amino)-1-oxopropan-2-yl) amino)-4-methyl-1-oxopentan-2-yl)amino)-4-methyl-1-oxopentan-2-yl dimethyl-L-isoleucinate (**18**).* A three-neck flask was charged with compound **17** (1.30 g, 2.15 mmol), Pd/C (500 mg) and methanol (60 mL). The resulting suspension was stirred for 2 h in hydrogen atmosphere. 37% formaldehyde solution (8 mL) was injected and the reaction mixture was stirred for further 24 h. The reaction mixture was filtered with diatomaceous earth. The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (petroleum ether: ethyl acetate= 5:1 to 2: 1) to afford desired compound **18** as a white solid (861 mg, 80%). $[\alpha]_D^{20}$ = – 78, (c = 1.0, MeOH). m. p. 82 °C. ν_{max} (KBr): 3303, 3080, 2961, 2874, 2784, 2376, 2310, 1739, 1670,1648, 1550, 1389, 1321, 1285, 1251, 1127 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 6.73 (d, J = 5.7 Hz, 1H), 6.51 (d, J = 6.8 Hz, 1H), 5.29 – 5.09 (m, 1H), 4.85 (s, 1H), 4.48 (s, 1H), 3.74 (s, 3H), 3.20 (s, 3H), 2.93 (d, J = 10.1 Hz, 1H), 2.30 (s, 6H), 1.77 (dd, J = 25.3, 13.0 Hz, 3H), 1.58 (d, J = 28.0 Hz, 4H), 1.30 (d, J = 6.2 Hz, 3H), 1.18 – 1.10 (m, 1H), 0.89 (dd, J = 17.8, 6.4 Hz, 19H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.6, 170.9, 170.8, 170.1, 72.5, 72.4, 61.7, 51.3, 45.8, 41.8, 41.6, 41.0, 33.5, 32.3, 25.1, 24.7, 24.5, 23.3, 23.1, 22.1, 21.5, 18.3, 15.8, 10.5. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{25}H_{48}N_4O_6Na^+$, 523.3466; found, 523.3464.

*(S)-4-Methyl-1-(((S)-4-methyl-1-oxo-1-(((S)-1-oxopropan-2-yl)amino)pentan-2-yl)amino)-1-oxopentan-2-yl dimethyl-L-isoleucinate (**4**).* A three-neck flask was charged with compound **18** （290 mg, 0.58 mmol）and dry THF (6 mL). The resulting solution cooled to – 78 °C in a nitrogen atmosphere. DIBAL-H (1.00 mL, 1.50 mmol) was added dropwise over 5 min and the reaction mixture was stirred for 4 h. the reaction mixture was quenched with MeOH (0.20 mL). The reaction mixture was poured into a saturated solution of sodium potassium tartrate (200 mL), added with diethyl ether (200 mL) and stirred for about 1 hour. The mixture was separated and the aqueous phase was extracted with ethyl acetate (3 × 100 mL). The organic phase was combined and dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo* to afford aldehyde **4**, which could be used directly without any further purification

*(S)-1-(((S)-1-(((S,E)-5-((S)-3-Methoxy-2-methyl-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-5-oxopent-3-en-2-yl)amino)-4-methyl-1-oxopentan-2-yl)amino)-4-methyl-1-oxopentan-2-yl dimethyl-L-isoleucinate (**1**).* A three-neck flask was charged with phosphonate ester **5** (0.19 g, 0.60 mmol) and dry THF (4 mL). The resulting solution cooled to – 78 °C in a nitrogen atmosphere. LiHMDS (0.60 mL, 0.60 mmol) was added dropwise. The reaction mixture was stirred for 15 min. A solution of aldehyde **4** (180 mg, 0.41 mmol, in 2 mL THF) was then added dropwise over 5 min. The resulting solution was stirred for further 3 h. After the substrate was completely consumed (monitored by TLC analysis), the reaction mixture was quenched with H_2O, and extracted by ethyl acetate (3 × 10 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (petroleum ether: ethyl acetate= 5:1) to afford desired compound **1** as a white solid (146 mg, 60%). $[\alpha]_D^{20}$ = – 57, (c

= 1.0, MeOH). m. p. 64 °C. v_{max}(KBr): 3301, 3079, 2962, 2936, 2873, 2789, 2352, 1731, 1652, 1630, 1549, 1455, 1382, 1327, 1291, 1260, 1225, 1056, 807 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41 (dd, *J* = 15.5, 1.4 Hz, 1H), 6.96 (dd, *J* = 15.5, 5.1 Hz, 1H), 6.41 (d, *J* = 8.2 Hz, 1H), 6.17 (d, *J* = 8.2 Hz, 1H), 5.15 (dd, *J* = 9.5, 4.0 Hz, 1H), 5.03 (s, 1H), 4.71 (dd, *J* = 12.8, 6.2 Hz, 1H), 4.61 (q, *J* = 6.5 Hz, 1H), 4.42 (td, *J* = 8.7, 5.5 Hz, 1H), 3.86 (s, 3H), 2.93 (d, *J* = 10.3 Hz, 1H), 2.31 (s, 6H), 1.74 (td, *J* = 13.2, 12.0, 4.6 Hz, 4H), 1.69 – 1.59 (m, 4H), 1.48 (d, *J* = 6.6 Hz, 3H), 1.29 (d, *J* = 7.0 Hz, 3H), 1.14 (dt, *J* = 22.2, 7.6 Hz, 1H), 1.03 – 0.79 (m, 18H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 180.8, 171.2, 170.7, 170.6, 169.8, 164.3, 148.3, 122.5, 93.1, 72.6, 58.9, 55.8, 51.6, 46.3, 41.7, 41.0, 40.8, 33.5, 25.1, 24.8, 24.5, 23.3, 23.0, 22.0, 21.4, 20.0, 17.1, 15.7, 10.5. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{31}H_{52}N_4O_7Na^+$, 615.3728; found, 615.3726.

*Methyl(S,E)-4-((S)-2-((S)-2-((dimethyl-L-isoleucyl)oxy)-4-methylpentanamido)-4-methylpentanamido) pent-2-enoate (**19**).* A round-bottom flask was charged with aldehyde **4** (100 mg, 0.23 mmol), methyl (triphenylphosphoranylidene) acetate (151 mg, 0.45 mmol) and dichloromethane (2 mL). The resulting solution was stirred overnight. The reaction mixture was concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (petroleum ether: ethyl acetate= 10:1 to 5: 1) to afford desired compound **19** as a white solid (109 mg, 95%) $[\alpha]_D^{20}$ = – 57.2, (c = 1.0, MeOH). m. p.112 °C. v_{max}(KBr): 3314, 3295, 3064, 2958, 2873, 2377, 2310, 1737, 1728, 1652, 1546, 1455, 1347, 1279, 1253, 1226, 1145, 1059 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.85 (dd, *J* = 15.7, 5.0 Hz, 1H), 6.65 (d, *J* = 8.0 Hz, 1H), 6.45 (d, *J* = 8.1 Hz, 1H), 5.88 (dd, *J* = 15.7, 1.6 Hz, 1H), 5.10 (dd, *J* = 9.5, 3.9 Hz, 1H), 4.63 (h, *J* = 6.8 Hz, 1H), 4.45 (td, *J* = 8.6, 5.6 Hz,1H), 3.71 (s, 3H), 2.93 (d, *J* = 10.3 Hz, 1H), 2.29 (s, 6H), 1.87 – 1.80 (m, 1H), 1.67 (ddt, *J* = 31.7, 12.8, 7.6 Hz, 6H), 1.23 (d, *J* = 7.0 Hz, 3H), 1.13 (dd, *J* = 14.2, 7.7 Hz, 1H), 0.96 – 0.82 (m, 19H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.3, 170.8, 170.7, 166.9, 148.7, 120.2, 72.6, 72.5, 51.7, 51.5, 45.8, 41.7, 40.9, 40.8, 33.4, 25.1, 24.9, 24.5, 23.3, 23.1, 22.0, 21.4, 19.8, 15.9, 10.5. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{26}H_{47}N_3O_6\ Na^+$, 520.3357; found, 520.3356.

*(3S,6S,9S,12S,E)-3-((S)-Sec-butyl)-6,9-diisobutyl-2,12-dimethyl-4,7,10-trioxo-5-oxa-2,8,11-triazapentadec-13-en-15-oic acid (**20**).* A round-bottom flask was charged with compound **19** (1.00 g, 2.01 mmol), LiOH (253 mg, 6.03 mmol) and aqueous tetrahydrofuran solution (20 mL, H_2O: THF= 1:1). 1 N hydrochloric acid solution was added to maintain pH of 4. The mixture was extracted with ethyl acetate. The combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo* to afford acid **20**, which could be used directly without any further purification.

*(S)-1-(((S)-1-(((S,E)-5-(Tert-butylamino)-5-oxopent-3-en-2-yl)amino)-4-methyl-1-oxopentan-2-yl)amino)-4-methyl-1-oxopentan-2-yl dimethyl-L-isoleucinate (**21a**).* A round-bottom flask was charged with acid **20** (200 mg, 0.41mmol), tert-butylamine (36.6 mg, 0.50 mmol), HOBT (67.6 mg, 0.5 mmol), DIPEA (265 mg, 2.05 mmol), EDCI (95.9 mg, 0.50 mmol) and dichloromethane (5 mL). The resulting suspension was stirred overnight. The reaction mixture was washed with 1 N hydrochloric acid solution, saturated sodium carbonate solution, and extracted by ethyl acetate. The combined organic phase were dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo.* The residue was purified by flash column chromatography on silica gel (petroleum ether:

ethyl acetate= 3:1 to 1: 1) to afford desired compound **21a** as a pale yellow oil (143 mg, 65%). $[\alpha]_D^{20}$ = – 39, (c = 1.0, MeOH). v_{max}(KBr): 3309, 3077, 2964, 2934, 2873, 2790, 1734, 1658, 1553, 1455, 1392, 1365, 1259, 1028, 800 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 6.65 (dd, *J* = 15.2, 5.1 Hz, 1H), 6.30 (dd, *J* = 20.3, 7.9 Hz, 1H), 5.78 (d, *J* = 15.0 Hz, 1H), 5.52 (s, 1H), 5.16 – 5.04 (m, 1H), 4.60 (d, *J* = 7.0 Hz, 1H), 4.45 – 4.30 (m, 1H), 2.95 (d, *J* = 10.3 Hz, 1H), 2.31 (s, 6H), 1.82 – 1.72 (m, 4H), 1.70 – 1.59 (m, 4H), 1.37 (s, 9H), 1.23 (d, *J* = 7.6 Hz, 5H), 0.98 – 0.84 (m, 18H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.5, 169.9, 163.7, 155.9, 141.8, 123.1, 71.8, 71.4, 52.4, 50.8, 50.3, 44.8, 41.1, 40.6, 39.7, 39.4, 32.4, 27.7, 24.0, 23.8, 23.5, 22.5, 22.5,22.2, 20.6, 20.3, 19.1, 17.2, 14.7, 9.3. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{29}H_{54}N_4O_5Na^+$, 561.3986; found, 561.3984.

*(S)-1-(((S)-1-(((S,E)-5-(Diethylamino)-5-oxopent-3-en-2-yl)amino)-4-methyl-1-oxopentan-2-yl)amino)-4-methyl-1-oxopentan-2-yl dimethyl-L-isoleucinate (**21b**).* The titled compound **21b** was obtained following the procedure described for **21a**. Flash column chromatography (petroleum ether: ethyl acetate= 5:1 to 1: 1). 115 mg, 52%, a pale yellow solid. $[\alpha]_D^{20}$ = – 48.5, (c = 1.0, MeOH). m. p. 63 °C. v_{max}(KBr): 3301, 3073, 2961, 2934, 2873, 2792, 2373, 1736, 1650, 1551, 1456, 1385, 1262, 1171, 1148, 1126, 1058, 980, 913 cm^{-1}. ^1H NMR (400 MHz, MeOD) δ 6.69 (dd, *J* = 15.0, 5.1 Hz, 1H), 6.37 (d, *J* = 15.2 Hz, 1H), 5.07 (d, *J* = 6.0 Hz, 1H), 4.62 – 4.54 (m, 1H), 4.48 – 4.40 (m, 1H), 3.44 (dt, *J* = 14.0, 7.1 Hz, 4H), 2.97 (d, *J* = 9.5 Hz, 1H), 2.30 (s, 6H), 1.83 – 1.75 (m, 3H), 1.67 (d, *J* = 10.1 Hz, 2H), 1.54 (d, *J* = 13.0 Hz, 2H), 1.29 (s, 7H), 1.21 (t, *J* = 7.0 Hz, 3H), 1.14 (t, *J* = 7.1 Hz, 3H), 0.99 – 0.86 (m, 18H). ^{13}C NMR (100 MHz, MeOD) δ 173.7, 172.8, 172.2, 167.5, 147.1, 120.7, 73.5, 73.4, 52.9, 47.4, 43.6, 42.4, 42.2, 42.0, 41.6, 34.7, 30.7, 26.2, 25.8, 25.6, 23.5, 21.9, 21.7, 20.0, 16.0, 15.1, 14.4, 13.2, 10.9. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{29}H_{54}N_4O_5Na^+$, 561.3986; found, 561.3985.

*(S)-4-methyl-1-(((S)-4-methyl-1-(((S,E)-5-morpholino-5-oxopent-3-en-2-yl)amino)-1-oxopentan-2-yl)amino)-1-oxopentan-2-yl dimethyl-L-isoleucinate (**21c**).* The titled compound **21c** was obtained following the procedure described for **21a**. Flash column chromatography (petroleum ether: ethyl acetate= 5:1 to 1: 1). 99.7 mg, 44%, as a white solid. $[\alpha]_D^{20}$ = – 46.7, (c = 1.0, MeOH). m. p.121 °C. v_{max}(KBr): 3335, 2959, 2871, 2787, 1732, 1645, 1532, 1436, 1385, 1366, 1263, 1173, 1117, 976, 916 cm^{-1}. ^1H NMR (400 MHz, MeOD) δ 6.67 (dd, *J* = 15.2, 5.6 Hz, 1H), 6.47 – 6.41 (d, 1H), 5.06 (dd, *J* = 9.8, 4.0 Hz, 1H), 4.61 – 4.53 (m, 1H), 4.39 (dd, *J* = 10.1, 5.2 Hz, 1H), 3.65 (m, *J* = 11.0, 5.1 Hz, 8H), 2.97 (d, *J* = 9.7 Hz, 1H), 2.30 (s, 6H), 1.80 (m, *J* = 17.4, 7.5 Hz, 3H), 1.66 (m, *J* = 15.0, 10.3, 5.1 Hz, 2H), 1.55 (m, *J* = 8.5, 4.5 Hz, 2H), 1.31 – 1.26 (m, 5H), 1.20 – 1.14 (m, 1H), 0.99 – 0.86 (m, 19H). ^{13}C NMR (100 MHz, MeOD) δ 173.7, 172.9, 172.3, 172.3, 147.5, 120.3, 73.5, 73.4, 67.8, 67.7, 53.1, 47.5, 43.6, 42.1, 42.0, 41.6, 34.7, 26.1, 25.8, 25.6, 23.6, 23.4, 21.9, 21.7, 19.9, 16.0, 10.9. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{29}H_{52}N_4O_6Na^+$, 575.3779; found, 575.3778.

*(S)-4-Methyl-1-(((S)-4-methyl-1-oxo-1-(((S)-1-oxopropan-2-yl)amino)pentan-2-yl)amino)-1-oxopentan-2-yl ((benzyloxy)carbonyl)-L-isoleucinate (**22**).* A three-neck flask was charged with compound **17** （390 mg, 0.64 mmol）and dry THF (6 mL). The resulting solution cooled to – 78 °C in a nitrogen atmosphere. DIBAL-H (0.64 mL, 0.96 mmol) was added dropwise over 5 min and the reaction mixture was stirred 4 h. the reaction mixture was quenched with MeOH (0.20 mL). The reaction mixture was poured into a saturated solution of sodium potassium tartrate (200 mL), added with diethyl ether (200 mL) and stirred for about 1 hour. The mixture was separated and

the aqueous phase was extracted with ethyl acetate (3 × 100 mL). The organic phase was combined and dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo* to afford aldehyde **22**, which could be used directly without any further purification.

*Diethyl(S)-(2-(2-benzyl-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-2-oxoethyl)phosphonate (**23**).* A round-bottom flask was charged with (*S*)-5-benzyl-1-(2-bromoacetyl)-4-methoxy-1,5-dihydro-2*H*-pyrrol-2-one (1.00 g, 3.10 mmol) and triethyl phosphite (2.60 g, 15.5 mmol). The resulting solution was stirred overnight in 55 °C. After the substrate was completely consumed (monitored by TLC analysis), the reaction mixture was concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (petroleum ether: ethyl acetate= 2:1 to dichloromethane: methanol=10: 1) to afford phosphonate ester **23** as a pale yellow oil (823 mg, 70%). $[\alpha]_D^{20}$ = +211.9, (c = 1.0, MeOH). ν_{max}(KBr): 3030, 2985, 1727, 1680, 1627, 1496, 1385, 1330, 1252, 1057, 1025, 970, 743, 703 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 7.13 (q, *J* = 6.9, 5.2 Hz, 3H), 6.97 – 6.90 (m, 2H), 4.81 – 4.78 (m, 1H), 4.76 (s, 1H), 4.12 (h, *J* = 7.8, 7.3 Hz, 4H), 3.83 – 3.76 (m, 1H), 3.74 (s, 3H), 3.70 – 3.62 (m, 1H), 3.46 (dd, *J* = 14.0, 4.9 Hz, 1H), 3.04 (dd, *J* = 14.0, 2.7 Hz, 1H), 1.26 (dt, *J* = 10.6, 7.1 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.9, 169.4, 164.2, 133.9, 129.5, 128.0, 126.9, 94.4, 62.3 , 59.8, 58.3, 36.0, 34.7, 34.4, 16.3. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{18}H_{24}NO_6PNa^+$, 404.1233; found, 404.1230.

*(S)-1-(((S)-1-(((S,E)-5-((S)-3-Methoxy-2-methyl-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-5-oxopent-3-en-2-yl)amino)-4-methyl-1-oxopentan-2-yl)amino)-4-methyl-1-oxopentan-2-yl ((benzyloxy)carbonyl)-L-isoleucinate (**24a**).* A three-neck flask was charged with phosphonate ester **5** (150 mg, 0.49) and dry THF (4 mL). The resulting solution cooled to – 78 °C in a nitrogen atmosphere. LiHMDS (0.50 mL,0.49) was added dropwise. The reaction mixture was stirred for 15 min. A solution of aldehyde **22** (180 mg, 0.33mmol, in 2 mL THF) was then added dropwise over 5 min. The resulting solution was stirred for further 3 h. After the substrate was completely consumed (monitored by TLC analysis), the reaction mixture was quenched with H_2O, and extracted by ethyl acetate (3 × 10 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (petroleum ether: ethyl acetate= 5:1 to 1: 1) to afford desired compound **24a** as a pale yellow oil (127 mg, 55%). $[\alpha]_D^{20}$ = – 14.3, (c = 1.0, MeOH). ν_{max}(KBr): 3292, 2963, 2873, 2377, 2310, 1732, 1680, 1456, 1338, 1291, 1094, 1025, 968, 807 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 7.40 – 7.29 (m, 8H), 6.97 (dd, *J* = 15.5, 5.4 Hz, 2H), 6.38 (d, *J* = 8.1 Hz, 1H), 5.26 (d, *J* = 6.2 Hz, 1H), 5.14 – 5.11 (m, 2H), 5.01 (s, 1H), 4.70 (q, *J* = 7.0 Hz, 1H), 4.60 (q, *J* = 6.5 Hz, 1H), 4.52 – 4.43 (m, 1H), 4.20 (dd, *J* = 6.5, 4.4 Hz, 1H), 3.84 (s, 3H), 3.74 (s, 1H), 3.19 (s, 1H), 1.98 (q, *J* = 8.3, 6.9 Hz, 1H), 1.89 (s, 1H), 1.70 (d, *J* = 5.9 Hz, 2H), 1.63 – 1.57 (m, 2H), 1.47 (d, *J* = 6.6 Hz, 3H), 1.30 (t, *J* = 6.4 Hz, 3H), 0.99 (dd, *J* = 7.0, 2.8 Hz, 3H), 0.94 – 0.87 (m, 15H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 179.5, 170.3, 169.9, 169.1, 168.6, 163.3, 156.1, 147.5, 134.8, 127.6, 127.4, 126.8, 121.4, 92.0, 73.4, 72.9, 66.2, 58.2, 57.8, 57.6, 54.6, 50.4, 45.1, 44.4, 39.8, 39.2, 35.9, 24.1, 23.8, 22.2, 20.3, 18.8, 17.0, 16.0, 14.8, 10.5. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{37}H_{54}N_4O_9Na^+$, 721.3783; found, 721.3781.

(S)-1-(((S)-1-(((S,E)-5-((S)-2-Benzyl-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-5-oxopent-3-en-2-

*yl)amino)-4-methyl-1-oxopentan-2-yl)amino)-4-methyl-1-oxopentan-2-yl ((benzyloxy)carbonyl)-L-isoleucinate (**24b**).* The titled compound **24b** was obtained following the procedure described for **24a**. Flash column chromatography (petroleum ether: ethyl acetate= 5:1 to 1: 1). 168 mg, 70%, as a yellow oil. $[\alpha]_D^{20}$ = + 64.5, (c = 1.0, MeOH). ν_{max}(KBr): 3308, 3064, 2958, 2873, 2350, 1730, 1681, 1632, 1537, 1348, 1329, 1027, 969,838.735 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 – 7.29 (m, 6H), 7.27 – 7.14 (m, 4H), 7.02 (dd, *J* = 15.8, 6.5 Hz, 1H), 6.94 (d, *J* = 6.1 Hz, 2H), 6.37 (d, *J* = 7.3 Hz, 1H), 5.23 (d, *J* = 5.0 Hz, 1H), 5.10 (m, *J* = 8.6 Hz, 2H), 4.88 (s, 1H), 4.79 (s, 1H), 4.74 (s, 1H), 4.46 (s, 1H), 4.21 (s,1H), 3.92 (s, 1H), 3.79 (s, 3H), 3.53 (dd, *J* = 13.8, 4.5 Hz, 1H), 3.10 (d, *J* = 13.4 Hz, 1H), 1.97 (s, 1H), 1.75 – 1.56 (m, 5H), 1.43 (m, *J* = 6.0 Hz, 1H), 1.30 (d, *J* = 6.5 Hz, 3H), 0.98 (d, *J* = 6.3 Hz, 3H), 0.92 – 0.89 (m, *J* = 12.4 Hz, 17H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.9, 171.4, 170.9, 170.3, 169.8, 164.7, 157.2, 149.0, 135.9, 134.4, 129.7, 128.7, 128.5, 128.3, 128.0, 127.1, 122.4, 95.0, 74.5, 67.3, 59.8, 59.3, 58.4, 51.6, 46.2, 40.4, 40.2, 37.1, 34.7, 25.2, 24.9, 24.7, 23.3, 23.3, 21.5, 20.0, 15.9, 11.7. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{43}H_{58}N_4O_9Na^+$, 797.4096; found, 797.4097.

*(S)-1-(((S)-1-(((S,E)-5-((S)-2-Isopropyl-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-5-oxopent-3-en-2-yl)amino)-4-methyl-1-oxopentan-2-yl)amino)-4-methyl-1-oxopentan-2-yl ((benzyloxy)carbonyl)-L-isoleucinate (**24c**).* The titled compound **24c** was obtained following the procedure described for **24a**. Flash column chromatography (petroleum ether: ethyl acetate= 5:1 to 1: 1). 85 mg, 65%, as a yellow oil. $[\alpha]_D^{20}$ = – 26.2, (c = 1.0, MeOH). ν_{max}(KBr): 3293, 2961, 2869, 2357, 2307, 1692, 1671, 1454, 1329, 1290, 1086, 1022, 966, 811 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 – 7.31 (m, 5H), 7.03 – 6.90 (m, 2H), 6.37 (d, *J* = 8.2 Hz, 1H), 5.21 (d, *J* = 6.4 Hz, 1H), 5.15 – 5.08 (m, 3H), 5.05 (d, *J* = 2.3 Hz, 1H), 4.71 (q, *J* = 7.2 Hz, 1H), 4.59 (d, *J* = 3.1 Hz, 1H), 4.46 (s, 1H), 4.21 (d, *J* = 5.8 Hz, 1H), 3.82 (s, 3H), 2.63 – 2.51 (m, 1H), 1.98 (s, 1H), 1.82 (s, 1H), 1.70 (s, 6H), 1.61 (d, *J* = 10.7 Hz, 1H), 1.49 – 1.40 (m, 1H), 1.34 – 1.23 (m, 6H), 1.10 (dd, *J* = 7.1, 2.2 Hz, 3H), 0.99 (d, *J* = 6.9 Hz, 3H), 0.95 – 0.88 (m, 12H), 0.74 (dd, *J* = 6.8, 2.2 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 179.6, 171.4, 171.0, 170.5, 170.3, 164.6, 157.2, 148.6, 135.9, 128.8, 128.6, 128.0, 122.8, 94.8, 74.6, 67.4, 64.0, 59.4, 58.6, 51.7, 46.3, 40.5, 40.2, 37.1, 29.8, 28.8, 25.3, 25.0, 24.8, 23.4, 23.3, 21.6, 21.5, 20.0, 19.0, 15.9, 15.5, 11.7. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{39}H_{58}N_4O_9Na^+$, 749.4096; found, 749.4099.

*Benzyl ((S)-1-(((S)-1-(methoxy(methyl)amino)-1-oxopropan-2-yl)amino)-4-methyl-1-oxopentan-2-yl)carbamate (**25**).* A round-bottom flask was charged with ((benzyloxy)carbonyl)-*L*-leucyl-*L*-alanine (329 mg, 0.98 mmol), N,O-dimethylhydroxylamine hydrochloride (287 mg, 2.94 mmol), HBTU (1.10 g, 2.94 mmol), DIPEA (633 mg, 4.90 mol) and THF(100 mL). The resulting suspension was stirred overnight. The reaction mixture was washed with 1 N hydrochloric acid solution, saturated sodium carbonate solution, and extracted by ethyl acetate. The combined organic phase were dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo.* The residue was purified by flash column chromatography on silica gel (petroleum ether: ethyl acetate= 3:1 to 1: 1) to afford weinreb amide **25** as a pale yellow oil (370 mg, 99.5%). $[\alpha]_D^{20}$ = – 19.8, (c = 1.0, MeOH). ν_{max} (KBr): 3370, 3035, 2958, 1722, 1650, 1537, 1322, 1082, 987, 851, 779 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.12 (d, *J* = 7.2 Hz, 1H), 7.47 – 7.25 (m, 6H), 5.02 (s, 2H), 4.77 – 4.64 (m, 1H), 4.08 (q, *J* = 8.7 Hz, 1H), 3.73 (s, 3H), 3.10 (s, 3H), 1.62 (dq, *J* = 13.1, 6.7 Hz, 1H), 1.41 (dd, *J* = 8.0, 4.7 Hz,

2H), 1.18 (dd, *J* = 10.5, 7.2 Hz, 3H), 0.86 (t, *J* = 5.8 Hz, 6H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.1, 155.9, 155.8, 137.1, 128.3, 127.8, 127.6, 127.6, 65.3, 61.1, 52.7, 44.6, 40.7, 31.7, 24.1, 23.1, 21.3, 17.0. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{19}H_{29}N_3O_5Na^+$, 402.1999; found, 402.1998.

*(S)-2-(dimethylamino)-N-((S)-1-(methoxy(methyl)amino)-1-oxopropan-2-yl)-4-methylpentanamide (**26**).* A three-neck flask was charged with compound **25** (380 mg, 1.00 mmol), Pd/C (200 mg) and methanol (25 mL). The resulting suspension was stirred for 2 h in hydrogen atmosphere. 37% formaldehyde solution (1 mL) was injected and the reaction mixture was stirred for further 24 h. The reaction mixture was filtered with diatomaceous earth. The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (petroleum ether: ethyl acetate= 3:1 to ethyl acetate) to afford desired compound **26** as a pale yellow oil (252 mg, 92%). $[\alpha]_D^{20}$ = – 11.6, (c = 1.0, MeOH). ν_{max}(KBr): 3334, 2957, 2870, 1664, 1468, 1389,1181, 1139, 1050, 993 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.43 (d, *J* = 7.1 Hz, 1H), 4.94 (m, *J* = 15.9, 9.5 Hz, 1H), 3.77 (s, 3H), 3.19 (s, 3H), 2.86 (dd, *J* = 8.2, 5.2 Hz, 1H), 2.26 (s, 6H), 1.69 (dq, *J* = 13.1, 6.6 Hz, 1H), 1.55 (m, 1H), 1.39 (td, *J* = 8.4, 4.3 Hz, 1H), 1.32 (d, *J* = 6.9 Hz, 3H), 0.91 (t, *J* = 6.7 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.3, 67.3, 61.8, 44.9, 42.3, 37.0, 25.9, 23.5, 22.3, 18.5. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{13}H_{27}N_3O_3Na^+$, 296.1945; found, 296.1943.

*(S)-2-(Dimethylamino)-4-methyl-N-((S)-1-oxopropan-2-yl) pentanamide (**27**).* A three-neck flask was charged with compound **26** (490 mg, 1.80 mmol) and dry THF (20 mL). Resulting suspension cooled to – 20 °C in a nitrogen atmosphere. LAH (75.1 mg, 1.98 mmol) was added and the reaction mixture was stirred 30 min. the reaction mixture was quenched with ethyl acetate (20 mL). The reaction mixture was poured into 0.1 N hydrochloric acid solution (30 mL). The mixture was separated and the aqueous phase was extracted with ethyl acetate. The organic phase was combined and dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo* to afford aldehyde **27**, which could be used directly without any further purification.

*(S)-2-(Dimethylamino)-N-((S,E)-5-((S)-3-methoxy-2-methyl-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-5-oxo pent-3-en-2-yl)-4-methylpentanamide (**28a**).* A three-neck flask was charged with phosphonate ester **5** (320 mg, 1.05 mmol) and dry THF (4 mL). The resulting solution cooled to – 78 °C in a nitrogen atmosphere. LiHMDS (1.05 mL, 1.05 mmol) was added dropwise. The reaction mixture was stirred for 15 min. A solution of aldehyde **27** (250 mg, 0.7 mmol, in 2 mL THF) was then added dropwise over 5 min. The resulting solution was stirred for further 3 h. After the substrate was completely consumed (monitored by TLC analysis), the reaction mixture was quenched with H_2O, and extracted by ethyl acetate (3 × 10 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (dichloromethane: methanol = 30:1 to 15: 1) to afford desired compound **28a** as a pale yellow oil. (179 mg, 70%). $[\alpha]_D^{20}$ = +19.29, (c = 1.0, MeOH). ν_{max}(KBr): 3300, 2955, 2870, 1727, 1680, 1625, 1504, 1455, 1326, 1293, 1247, 1185, 1132, 950, 807 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41 (dd, *J* = 15.4, 11.5 Hz, 1H), 7.21 (s, 1H), 7.03 (td, *J* = 15.4, 4.4 Hz, 1H), 5.02 (d, *J* = 3.0 Hz, 1H), 4.78 (d, *J* = 8.9 Hz, 1H), 4.68 – 4.56 (m, 1H), 3.86 (s, 3H), 2.97 (s, 1H), 2.32 (d, *J* = 13.1 Hz, 6H), 1.82 – 1.56 (m, 3H), 1.48 (dd, *J* = 6.7, 2.9 Hz, 3H), 1.34 – 1.28 (m, 3H), 0.93 (q, *J* = 6.2, 5.4 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 179.7, 168.7, 163.3, 148.4, 147.9, 121.1,

120.6, 92.0, 66.0, 57.7, 54.7, 44.4, 41.1, 35.2, 24.9, 22.4, 21.1, 19.2, 16.1. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{19}H_{31}N_3O_4Na^+$, 388.2207; found, 388.2206.

*(S)-N-((S,E)-5-((S)-2-Benzyl-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-5-oxopent-3-en-2-yl)-2-(dimethylamino)-4-methylpentanamide (**28b**).* The titled compound **28b** was obtained following the procedure described for **28a**. Flash column chromatography (dichloromethane: methanol = 40:1 to 15: 1). 124 mg, 85%, as a pale yellow oil. $[\alpha]_D^{20}$ = +73.9, (c = 1.0, MeOH). ν_{max}(KBr): 3320, 2955, 2869, 2829, 1729, 1679, 1629, 1499, 1454, 1329, 1194, 1169, 1057, 971, 810, 742, 703 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 8.08 (dd, *J* = 28.1, 6.9 Hz, 1H), 7.20 (q, *J* = 6.1 Hz, 4H), 7.03 – 6.94 (m, 1H), 6.89 – 6.78 (m, 2H), 5.15 (d, *J* = 3.4 Hz, 1H), 4.95 (s, 1H), 4.61 (q, *J* = 6.0 Hz, 1H), 3.84 (s, 3H), 3.73 (s, 1H), 3.10 (s, 1H), 3.03 (d, *J* = 13.8 Hz, 1H), 2.25 – 2.11 (m, 6H), 1.56 – 1.46 (m, 2H), 1.38 – 1.29 (m, 1H), 1.30 – 1.17 (m, 3H), 0.85 (dq, *J* = 9.7, 4.6 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.9, 169.8, 164.7, 149.4, 134.3, 129.6, 128.2, 127.1, 121.9, 95.0, 67.2, 61.7, 59.8, 58.4, 45.0, 42.2, 37.0, 34.6, 25.8, 23.4, 22.2, 20.4, 18.4. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{25}H_{35}N_3O_4Na^+$, 464.2520; found, 464.2518.

*(S)-N-((S,E)-5-((R)-2-Benzyl-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-5-oxopent-3-en-2-yl)-2-(dimethylamino)-4-methylpentanamide (**28c**).* The titled compound **28c** was obtained following the procedure described for **28a**. Flash column chromatography (dichloromethane: methanol = 40:1 to 15: 1). 120 mg, 82%, as a pale yellow oil. $[\alpha]_D^{20}$ = –54.6, (c = 1.0, MeOH). ν_{max}(KBr): 3320, 2954, 2869, 2829, 1725, 1658, 1619, 1479, 1434, 1329, 1164, 1119, 1051, 901, 815, 722, 709 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 7.36 (d, *J* = 15.5 Hz, 1H), 7.23 – 7.08 (m, 5H), 6.98 – 6.92 (m, 2H), 6.20 (d, *J* = 21.1 Hz, 1H), 4.90 (t, *J* = 3.9 Hz, 1H), 4.81 (d, *J* = 2.7 Hz, 1H), 4.04 (dd, *J* = 38.8, 8.1 Hz, 1H), 3.82 (s, 3H), 3.56 (dd, *J* = 14.0, 5.0 Hz, 1H), 3.25 – 3.07 (m, 1H), 2.33 (s, 6H), 1.70 – 1.63 (m, 2H), 1.52 (d, *J* = 6.9 Hz, 1H), 1.35 (d, *J* = 6.9 Hz, 2H), 1.26 (s, 1H), 0.94 (t, *J* = 6.0 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.9, 173.1, 169.8, 164.6, 149.4, 134.2, 129.6, 128.2, 127.0, 121.8, 94.9, 67.1, 59.7, 58.3, 45.3, 44.8, 42.2, 36.9, 25.7, 23.3, 22.1, 20.3, 18.3. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{25}H_{35}N_3O_4Na^+$, 464.2520; found, 464.2518.

*(S)-2-(Dimethylamino)-N-((S,E)-5-((S)-2-isopropyl-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-5-oxopent-3-en-2-yl)-4-methylpentanamide (**28d**).* The titled compound **28d** was obtained following the procedure described for **28a**. Flash column chromatography (dichloromethane: methanol = 40:1 to 15: 1). 108 mg, 60%, as a pale yellow oil. $[\alpha]_D^{20}$ = +28.46, (c = 1.0, MeOH). ν_{max}(KBr): 3311, 2943, 2874, 1736, 1661, 1623, 1500, 1452, 1313, 1291, 1242, 1178, 1121, 950, 809 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 7.41 (d, *J* = 15.5 Hz, 1H), 6.98 (dd, *J* = 15.5, 5.2 Hz, 1H), 5.06 (s, 1H), 4.78 (q, *J* = 7.1 Hz, 1H), 4.60 (s, 1H), 3.84 (s, 3H), 2.56 (tt, *J* = 7.3, 4.5 Hz, 1H), 2.36 (s, 6H), 1.71 (ddd, *J* = 27.0, 17.1, 10.3 Hz, 2H), 1.51 (d, *J* = 7.0 Hz, 1H), 1.42 (dt, *J* = 9.9, 4.9 Hz, 1H), 1.32 (d, *J* = 6.8 Hz, 3H), 1.25 (s, 1H), 1.11 (d, *J* = 7.2 Hz, 3H), 1.00 – 0.89 (m, 6H), 0.74 (d, *J* = 7.0 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 179.8, 170.6, 164.6, 148.9, 122.4, 94.8, 67.0, 64.1, 58.6, 45.7, 42.1, 36.4, 29.8, 28.9, 26.0, 23.6, 22.2, 20.3, 19.0, 15.5. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{21}H_{35}N_3O_4Na^+$, 416.2520; found, 416.2522.

*((Benzyloxy)carbonyl)-L-isoleucyl-L-leucyl-L-leucyl-L-alanine (**29**).*

Resin loading: Commercially available 2-CTC resin (1.0 g, 1.0 mmol/g) was pre-swelled for 20 min

in DCM in a manual solid phase peptide synthesis vessel (100 mL). DCM (20 mL), DIPEA(0.4 g，3.0 mmol) and Fmoc-Ala-OH (0.6 g, 2.0 mmol) were added to the resin. The mixture was agitated for 1 h before the unreacted resin was capped with Blocking fluid (DCM: MeOH: DIPEA= 17: 2: 1). Then the solvent was drained, and the resin was washed with DCM (5 × 20 mL) and DMF (5 × 20 mL). The resin loading was determined to be 1 mmol/g in the case of complete reaction of Fmoc-Ala-OH with the resin.

Peptide elongation: After the first amino acid was loaded on the resin, the peptide chain was elongated by amino acid additions until the linear precursor of acid **29** was obtained. Each amino acid addition involved deprotection and coupling procedures. The methods of deprotection, coupling of amino acids were described as follows:

Method A: Fmoc removal

A solution of 20% piperidine in DMF (20 mL) was added to the resin and the resulting suspension was shaken for 30 min. The solution was drained and the resin was washed with DMF (5 × 20 mL).

Method B: Coupling of amino acids

Fmoc-*L*-Leucine (1.5 eq.) and HBTU (1.5 eq.) were dissolved in DMF (20 mL). DIEA (3 eq.) was added to the resulting solution. The solution was stirred for 5 min at room temperature before it was transferred to the deprotected peptidyl resin. The mixture was agitated for 2 h until a negative chloranil test was observed. Then the solvent was drained and the resin was rinsed with DMF (5 × 20 mL). Apply the same method to couple the second Fmoc-L-Leucine amino acids. Then N-Cbz-*L*-Isoleucine (3 eq.) and HBTU (3 eq.) were dissolved in DMF (20 mL). DIEA (6 eq.) was added to the resulting solution. The solution was stirred for 5 min at room temperature before it was transferred to the deprotected peptidyl resin. The mixture was agitated for 2 h until a negative chloranil test was observed. Then the solvent was drained and the resin was rinsed with DMF (5 × 20 mL).

Resin cleavage: After all of the amino acids were coupled to the resin, The peptidyl resin was washed with DCM (5 × 20 mL) and was then suspended in 1% TFA/DCM (20 mL) and the mixture was shaken at room temperature for 30 min. Repeat the above process for five times. Then the resin was removed by filtration and washed with DCM (20 mL). The filtrate was concentrated *in vacuo* to afford acid **29**, which could be used directly without any further purification.

*Benzyl ((5S,8S,11S,14S,15S)-8,11-diisobutyl-3,5,15-trimethyl-4,7,10,13-tetraoxo-2-oxa-3,6,9,12-tetraazaheptadecan-14-yl)carbamate (**30**).* A round-bottom flask was charged with acid **29** (300 mg, 0.53 mmol), N,O-dimethylhydroxylamine hydrochloride (208 mg, 2.10 mmol), HBTU (796 mg, 2.10 mmol), DIPEA (0.44 mL, 2.65 mmol) and DCM (6 mL). The resulting suspension was stirred overnight. The reaction mixture was washed with 1 N hydrochloric acid solution, saturated sodium carbonate solution, and extracted by ethyl acetate. The combined organic phase were dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo.* The residue was purified by flash column chromatography on silica gel (petroleum ether: ethyl acetate= 1:1) to afford weinreb amide **30** as a white solid (254 mg, 79%). $[\alpha]_D^{20}$ = – 29.9, (c = 1.0, MeOH). m. p. 183 °C. ν_{max}(KBr): 3286, 3069, 2961, 2873, 1686, 1545, 1387, 1236, 1092, 991, 780, 737 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 8.01 (d, *J* = 29.5 Hz, 3H), 7.29 (dd, *J* = 12.8, 5.2 Hz, 5H), 6.53 (s, 1H), 5.19 – 5.00 (m, 3H), 5.02 - 4.82 (m, 2H), 4.27 (t, *J* = 8.0 Hz, 1H), 3.70 (s, 3H), 3.17 (s, 3H), 1.81 (d, *J* = 6.3 Hz, 1H), 1.67 – 1.51 (m, 7H), 1.27 (d, *J* = 6.9 Hz, 3H), 1.09 (dt, *J* = 14.3, 7.8 Hz, 1H), 0.89 – 0.80 (m, 18H). ^{13}C NMR

(100 MHz, $CDCl_3$) δ 173.0, 172.3, 172.0, 156.7, 136.8, 128.4, 127.9, 127.8, 66.7, 61.6, 59.8, 51.7, 45.1, 41.8, 41.6, 37.9, 32.2, 29.8, 25.1, 24.9, 24.8, 22.9, 22.7, 22.5, 17.9, 15.4, 11.4. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{31}H_{51}N_5O_7Na^+$, 628.3681; found, 628.3680.

*(2S,3S)-2-(dimethylamino)-N-((5S,8S,11S)-8-isobutyl-3,5,13-trimethyl-4,7,10-trioxo-2-oxa-3,6,9-triazatetradecan-11-yl)-3-methylpentanamide (**31**).* A three-neck flask was charged with compound **30** (1.00 g, 1.65 mmol), Pd/C (500 mg) and methanol (50 mL). The resulting suspension was stirred for 2 h in hydrogen atmosphere. 37% formaldehyde solution (4 mL) was injected and the reaction mixture was stirred for further 24 h. The reaction mixture was filtered with diatomaceous earth. The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (petroleum ether: ethyl acetate= 5:1 to 4: 1) to afford desired compound **31** as a white solid (701 mg, 85%). $[\alpha]_D^{20}$ = – 45.5, (c = 1.0, MeOH). m. p. 102 °C. ν_{max}(KBr): 3284, 3078, 2961, 2874, 2377, 2310, 1630, 1539, 1369, 844 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 7.13 (d, *J* = 6.6 Hz, 1H), 7.00 (d, *J* = 8.2 Hz, 1H), 6.81 (d, *J* = 7.8 Hz, 1H), 4.98 – 4.86 (m, 1H), 4.54 (p, *J* = 8.0 Hz, 2H), 3.74 (s, 3H), 3.18 (s, 3H), 2.58 (d, *J* = 5.8 Hz, 1H), 2.22 (s, 6H), 1.85 – 1.75 (m, 1H), 1.56 – 1.47(m, 7H), 1.28 (d, *J* = 6.8 Hz, 3H), 1.14 (dt, *J* = 14.4, 7.5 Hz, 1H), 0.91 – 0.86 (m, 19H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.9, 172.1, 171.8, 171.4, 74.3, 61.7, 51.6, 51.0, 45.5, 43.0, 41.4, 41.1, 34.3, 26.7, 24.8, 24.6, 23.1, 22.0, 21.9, 18.2, 14.8, 11.9. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{25}H_{49}N_5O_5Na^+$, 522.3626; found, 522.3625.

*(2S,3S)-2-(Dimethylamino)-3-methyl-N-((S)-4-methyl-1-(((S)-4-methyl-1-oxo-1-(((S)-1-oxopropan-2-yl)amino)pentan-2-yl)amino)-1-oxopentan-2-yl)pentanamide (**32**).* A three-neck flask was charged with compound **31** (200 mg, 0.40 mmol) and dry THF (6 mL). Resulting suspension cooled to – 20 °C in a nitrogen atmosphere. LAH (18.2 mg, 0.48 mmol) was added and the reaction mixture was stirred 30 min. the reaction mixture was quenched with ethyl acetate (20 mL). The reaction mixture was poured into 0.1 N hydrochloric acid solution (20 mL). The mixture was separated and the aqueous phase was extracted with ethyl acetate. The organic phase was combined and dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo* to afford aldehyde **32**, which could be used directly without any further purification.

*(2S,3S)-2-(Dimethylamino)-N-((S)-1-(((S)-1-(((S,E)-5-((S)-3-methoxy-2-methyl-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-5-oxopent-3-en-2-yl)amino)-4-methyl-1-oxopentan-2-yl)amino)-4-methyl-1-oxopentan-2-yl)-3-methylpentanamide (**33a**).* A three-neck flask was charged with phosphonate ester **5** (312 g, 1.02 mmol) and dry THF (4 mL). The resulting solution cooled to – 78 °C in a nitrogen atmosphere. LiHMDS (1.00 mL , 1.02 mmol) was added dropwise. The reaction mixture was stirred for 15 min. A solution of aldehyde **32** (300 mg, 0.68 mmol, in 2 mL THF) was then added dropwise over 5 min. The resulting solution was stirred for further 3 h. After the substrate was completely consumed (monitored by TLC analysis), the reaction mixture was quenched with H_2O, and extracted by ethyl acetate (3 × 10 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (dichloromethane: methanol = 60:1 to 15: 1) to afford desired compound **33a** as a pale yellow oil (181 mg, 45%). $[\alpha]_D^{20}$ = – 37, (c = 1.0, MeOH). ν_{max}(KBr): 3288, 3072, 2959, 2936, 2873, 2787, 1729, 1642, 1626, 1547, 1454, 1356, 1247, 1029, 986 cm^{-1}. ^1H

NMR (400 MHz, DMSO-d_6) δ 8.16 (d, J = 7.8 Hz, 1H), 7.93 (d, J = 8.4 Hz, 1H), 7.82 (d, J = 8.3 Hz, 1H), 7.27 (dd, J = 15.5, 1.7 Hz, 1H), 6.87 (dd, J = 15.5, 4.9 Hz, 1H), 5.34 (s, 1H), 4.62 (q, J = 6.6 Hz, 1H), 4.51 (dq, J = 10.9, 5.3, 3.7 Hz, 1H), 4.42 – 4.34 (m, 1H), 3.99 (dtd, J = 14.7, 7.4, 3.2 Hz, 1H), 3.86 (s, 3H), 2.67 (d, J = 10.1 Hz, 1H), 2.15 (d, J = 2.8 Hz, 6H), 1.78 – 1.69 (m, 1H), 1.65 – 1.54 (m, 2H), 1.48 (dd, J = 9.3, 5.5 Hz, 2H), 1.37 (d, J = 6.7 Hz, 3H), 1.18 (d, J = 7.0 Hz, 3H), 1.10 – 1.01 (m, 1H), 0.91 – 0.79 (m, 18H), 0.72 (d, J = 6.7 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 180.7, 171.8, 171.1, 169.5, 169.3, 163.3, 149.5, 121.1, 93.0, 71.1, 59.2, 54.8, 50.8, 50.4, 45.2, 41.4, 41.3, 40.4, 32.4, 24.7, 24.1, 23.1, 23.0, 21.5, 21.1, 19.5, 16.7, 15.2, 10.4. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{31}H_{53}N_5O_6Na^+$, 614.3888; found, 614.3886.

*(2S,3S)-2-(Dimethylamino)-N-((S)-1-(((S)-1-(((S,E)-5-((S)-2-isopropyl-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-5-oxopent-3-en-2-yl)amino)-4-methyl-1-oxopentan-2-yl)amino)-4-methyl-1-oxopentan-2-yl)-3-methylpentanamide (**33b**).* The titled compound **33b** was obtained following the procedure described for **33a**. Flash column chromatography (dichloromethane: methanol = 60:1 to 15: 1). 153 mg, 55%, as a pale yellow oil. $[\alpha]_D^{20}$ = – 26, (c = 1.0, MeOH). ν_{max} (KBr): 3419, 2962, 2874, 1730, 1668, 1624, 1555, 1327, 1029, 995 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.17 (d, J = 7.8 Hz, 1H), 7.93 (d, J = 8.4 Hz, 1H), 7.82 (d, J = 8.4 Hz, 1H), 7.27 (dd, J = 15.5, 1.7 Hz, 1H), 6.85 (dd, J = 15.5, 4.9 Hz, 1H), 5.38 (s, 1H), 4.59 (d, J = 2.6 Hz, 1H), 4.51 (q, J = 6.6 Hz, 1H), 4.41 – 4.35 (m, 1H), 4.31 (q, J = 7.8 Hz, 1H), 3.85 (s, 3H), 2.67 (d, J = 10.0 Hz, 1H), 2.43 (pd, J = 7.1, 2.4 Hz, 1H), 2.16 (s, 6H), 1.72 (dd, J = 11.9, 5.7 Hz, 1H), 1.66 – 1.53 (m, 2H), 1.47 (q, J = 7.0 Hz, 2H), 1.38 (td, J = 8.7, 8.2, 4.3 Hz, 1H), 1.18 (d, J = 7.0 Hz, 3H), 1.05 (d, J = 7.1 Hz, 3H), 0.92 – 0.78 (m, 18H), 0.72 (d, J = 6.6 Hz, 3H), 0.66 (d, J = 6.9 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 179.6, 171.9, 171.2, 169.9, 169.6, 163.4, 149.6, 121.1, 94.8, 71.0, 63.1, 59.1, 50.8, 50.4, 45.2, 41.4, 40.3, 32.4, 28.1, 24.7, 24.1, 23.2, 23.1, 21.5, 21.1, 19.5, 18.6, 15.3, 15.1, 10.4. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{33}H_{57}N_5O_6Na^+$, 642.4201; found, 642.4200.

*Benzyl ((2S,3S)-3-methyl-1-(((S)-4-methyl-1-(((S)-4-methyl-1-oxo-1-(((S)-1-oxopropan-2-yl)amino)pentan-2-yl)amino)-1-oxopentan-2-yl)amino)-1-oxopentan-2-yl)carbamate (**34**).* A three-neck flask was charged with compound **30** (200 mg, 0.33 mmol) and dry THF (5 mL). Resulting suspension cooled to – 20 °C in a nitrogen atmosphere. LAH (15.0 mg, 0.37 mmol) was added and the reaction mixture was stirred 30 min. the reaction mixture was quenched with ethyl acetate (10 mL). The reaction mixture was poured into 0.1 N hydrochloric acid solution (10 mL). The mixture was separated and the aqueous phase was extracted with ethyl acetate. The combined organic layers was combined and dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo* to afford aldehyde **34**, which could be used directly without any further purification.

*Benzyl ((2S,3S)-1-(((S)-1-(((S)-1-(((S,E)-5-((S)-3-methoxy-2-methyl-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-5-oxopent-3-en-2-yl)amino)-4-methyl-1-oxopentan-2-yl)amino)-4-methyl-1-oxopentan-2-yl)amino)-3-methyl-1-oxopentan-2-yl)carbamate (**35a**).* A three-neck flask was charged with phosphonate ester **5** (168 mg, 0.55 mmol) and dry THF. The resulting solution cooled to – 78 °C in a nitrogen atmosphere. LiHMDS (0.55 mL, 0.55mmol) was added dropwise. The reaction mixture was stirred for 15 min. A solution of aldehyde **34** (200 mg, 0.37 mmol, in THF 2 mL) was then added dropwise

over 5 min. The resulting solution was stirred for further 3 h. After the substrate was completely consumed (monitored by TLC analysis), the reaction mixture was quenched with H_2O, and extracted by ethyl acetate. The combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (ethyl acetate: methanol = 50:1) to afford desired compound **35a** as a white solid (186 mg, 72%). $[\alpha]_D^{20}$ = – 22, (c = 1.0, MeOH). m. p. 161 °C. ν_{max} (KBr): 3279, 3069, 2960, 2874, 2363, 1693, 1637, 1541, 1380, 1286, 1180, 1029 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 8.10 (d, *J* = 7.8 Hz, 1H), 7.96 (d, *J* = 8.4 Hz, 1H), 7.80 (d, *J* = 8.6 Hz, 1H), 7.38 – 7.28 (m, 7H), 6.86 (d, *J* = 15.3 Hz, 1H), 5.34 (s, 1H), 5.02 (s, 2H), 4.62 (dd, *J* = 6.9, 2.6 Hz, 1H), 4.50 (s, 1H), 4.32 (t, *J* = 8.2 Hz, 2H), 3.87 (s, 3H), 1.70 (s, 1H), 1.59 (q, *J* = 6.7 Hz, 2H), 1.50 (d, *J* = 7.0 Hz, 2H), 1.43 (d, *J* = 7.5 Hz, 2H), 1.37 (d, *J* = 5.8 Hz, 3H), 1.25 – 1.22 (m, 1H), 1.21 – 1.16 (m, 4H), 1.10 (q, *J* = 11.7, 9.8 Hz, 1H), 0.90 – 0.79 (m, 18H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 181.2, 171.9, 171.6, 171.4, 169.8, 163.7, 156.4, 149.9, 137.5, 128.7, 128.2, 128.0, 121.6, 93.5, 65.7, 59.7, 55.3, 51.2, 45.7, 41.6, 36.8, 24.8, 24.6, 24.5, 23.5, 23.4, 22.1, 21.9, 19.9, 17.2, 15.7, 11.3. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{37}H_{55}N_5O_8Na^+$, 720.3943; found, 720.3941.

*Benzyl ((2S,3S)-1-(((S)-1-(((S)-1-(((S,E)-5-((S)-2-isopropyl-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-5-oxopent-3-en-2-yl)amino)-4-methyl-1-oxopentan-2-yl)amino)-4-methyl-1-oxopentan-2-yl)amino)-3-methyl-1-oxopentan-2-yl)carbamate (**35b**).* The titled compound **35b** was obtained following the procedure described for **35a**. Flash column chromatography (dichloromethane: methanol = 40:1 to 15: 1). 116 mg, 43%, as a white solid. $[\alpha]_D^{20}$ = +3.8, (c = 1.0, MeOH). m. p. 125 °C. ν_{max}(KBr): 3290, 3070, 2962, 2935, 2874, 1728, 1641, 1537, 1455, 1344, 1321, 1242,1175, 1039, 995, 963 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 8.12 (d, *J* = 7.7 Hz, 1H), 7.98 (d, *J* = 8.2 Hz, 1H), 7.82 (d, *J* = 8.4 Hz, 1H), 7.40 – 7.22 (m, 7H), 6.85 (dd, *J* = 15.5, 4.9 Hz, 1H), 5.38 (s, 1H), 5.02 (s, 2H), 4.59 (d, *J* = 2.4 Hz, 1H), 4.51 (q, *J* = 6.5 Hz, 1H), 4.32 (p, *J* = 7.9 Hz, 2H), 3.89 (t, *J* = 8.2 Hz, 1H), 3.85 (s, 3H), 2.43 (dtd, *J* = 13.5, 6.8, 2.2 Hz, 1H), 1.70 (d, *J* = 6.0 Hz, 1H), 1.59 (dq, *J* = 13.2, 6.6 Hz, 2H), 1.52 – 1.46 (m, 2H), 1.46 – 1.40 (m, 2H), 1.23 (s, 1H), 1.18 (d, *J* = 6.9 Hz, 3H), 1.04 (d, *J* = 7.1 Hz, 3H), 0.91 – 0.76 (m, 19H), 0.66 (d, *J* = 6.9 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 179.6, 171.5, 171.1, 171.0, 170.0, 163.4, 156.0, 149.6, 137.1, 128.3, 127.7, 127.6, 121.2, 94.8, 65.3, 63.0, 59.1, 50.8, 45.2, 41.2, 40.7, 36.3, 28.1, 24.3, 24.1, 24.0, 23.1, 23.0, 21.6, 21.5, 19.5, 18.6, 15.3, 15.1, 10.9. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{39}H_{59}N_5O_8Na^+$, 748.4256; found, 748.4255.

*Tert-butyl 3-(((S)-1-((5S,8S,11S,14S,E)-5-((S)-sec-butyl)-8,11-diisobutyl-14-methyl-3,6,9,12-tetraoxo-1-phenyl-2-oxa-4,7,10,13-tetraazaheptadec-15-en-17-oyl)-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-2-yl)methyl)-1H-indole-1-carboxylate (**35c**).* The titled compound **35c** was obtained following the procedure described for **35a**. Flash column chromatography (dichloromethane: methanol = 40:1 to 15: 1). 331mg, 79%, as a white solid. $[\alpha]_D^{20}$ = +51.1, (c = 1.0, MeOH). m. p. 122 °C. ν_{max} (KBr): 3286, 3067, 2960, 2934, 2873, 2245, 1730, 1642, 1538, 1454, 1376, 1339, 1255, 1122, 1053, 969, 767, 748, 697 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 8.14 (d, *J* = 7.7 Hz, 1H), 7.97 (t, *J* = 9.2 Hz, 2H), 7.79 (d, *J* = 8.3 Hz, 1H), 7.33 (q, *J* = 6.6 Hz, 7H), 7.23 – 7.15 (m, 3H), 7.00 (dd, *J* = 15.4, 4.7 Hz, 1H), 5.18 (s, 1H), 5.01 (d, *J* = 7.3 Hz, 2H), 4.51 (q, *J* = 6.7 Hz, 1H), 4.33 (p, *J* = 7.6 Hz, 2H), 3.89 (t, *J* = 8.3 Hz, 1H), 3.79 (s, 3H), 3.55 (dd, *J* = 14.8, 5.1 Hz, 1H), 3.20 (d, *J* = 14.7 Hz, 1H), 1.69 (s, 1H), 1.60 (s,

11H), 1.45 (dt, *J* = 14.7, 7.7 Hz, 5H), 1.20 (q, *J* = 8.0 Hz, 3H), 1.14 – 1.05 (m, 1H), 0.87 – 0.77 (m, 20H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 178.4, 171.4, 171.1, 170.9, 169.5, 163.9, 155.9, 149.9, 148.8, 137.0, 134.2, 130.5, 128.2, 127.7, 127.5, 124.3, 124.1, 122.5, 120.8, 118.8, 114.6, 113.3, 94.8, 83.7, 65.3, 59.1, 58.9, 58.6, 50.8, 50.7, 45.2, 41.2, 40.7, 36.3, 27.6, 24.3, 24.1, 24.0, 23.6, 23.0, 22.9, 21.6, 21.4, 19.4, 15.2, 10.8. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{50}H_{68}N_6O_{10}Na^+$, 935.4889; found, 935.4888.

*Benzyl ((2S,3S)-1-(((S)-1-(((S)-1-(((S,E)-5-((S)-2-((1H-indol-3-yl)methyl)-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-5-oxopent-3-en-2-yl)amino)-4-methyl-1-oxopentan-2-yl)amino)-4-methyl-1-oxopentan-2-yl)amino)-3-methyl-1-oxopentan-2-yl)carbamate (**35d**).* A round-bottom flask was charged with compound **35c** (300 mg, 0.33 mmol) and dichloromethane (3 mL). The resulting solution was cooled to 0 °C. TFA (1.5 mL) was then added slowly. The reaction mixture was stirred 1 h. After the substrate was completely consumed (monitored by TLC analysis), the reaction mixture was wash with saturated sodium carbonate solution, and extracted by ethyl acetate. The combined organic phase were dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo.* The residue was purified by flash column chromatography on silica gel (petroleum ether: ethyl acetate= 3:1) to afford desired compound **35d** as a white solid (220 mg, 82%). $[\alpha]_D^{20}$ = + 41.1, (c = 1.0, MeOH). m. p. 118 °C. ν_{max}(KBr): 3287, 3067, 2960, 2933, 2873, 1726, 1642, 1539, 1456, 1351, 1285, 1123, 970, 807, 777 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.83 (s, 1H), 8.13 (d, *J* = 6.9 Hz, 1H), 7.95 (d, *J* = 7.7 Hz, 1H), 7.77 (d, *J* = 7.8 Hz, 1H), 7.35 (s, 4H), 7.29 (d, *J* = 7.7 Hz, 4H), 7.20 (d, *J* = 15.5 Hz, 1H), 7.05 – 6.89 (m, 3H), 6.80 (s, 1H), 5.05 (s, 1H), 5.02 (s, 2H), 4.93 (s, 1H), 4.51 (s, 1H), 4.38 – 4.28 (m, 2H), 3.90 (t, *J* = 7.7 Hz, 1H), 3.77 (s, 3H), 3.60 (d, *J* = 11.2 Hz, 1H), 3.20 (d, *J* = 14.7 Hz, 1H), 1.70 (s, 1H), 1.59 (d, *J* = 5.5 Hz, 2H), 1.45 (d, *J* = 6.4 Hz, 4H), 1.26 – 1.18 (m, 4H), 1.10 (d, *J* = 7.1 Hz, 1H), 0.88 – 0.76 (m, 18H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 178.7, 171.4, 171.0, 170.9, 169.6, 163.7, 155.9, 149.4, 137.0, 135.6, 128.2, 127.8, 127.6, 127.5, 123.8, 121.1, 120.7, 118.4, 117.8, 111.3, 106.3, 94.6, 65.2, 59.2, 59.1, 58.8, 50.8, 45.2, 41.1, 40.7, 36.3, 24.1, 24.0, 23.0, 21.6, 21.4, 19.4, 15.2, 10.8. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{45}H_{60}N_6O_8Na^+$, 835.4365; found, 835.4364.

*Benzyl ((2S,3S)-1-(((S)-1-(((S)-1-(((S,E)-5-((S)-2-benzyl-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-5-oxopent-3-en-2-yl)amino)-4-methyl-1-oxopentan-2-yl)amino)-4-methyl-1-oxopentan-2-yl)amino)-3-methyl-1-oxopentan-2-yl)carbamate (**35e**).* The titled compound **35e** was obtained following the procedure described for **35a**. Flash column chromatography (dichloromethane: methanol = 50:1 to 18: 1). 240 mg, 84%, as a white solid. $[\alpha]_D^{20}$ = + 45.7, (c = 1.0, MeOH). m. p. 202 °C. ν_{max}(KBr): 3283, 3067, 3032, 2960, 2934, 2873, 1729, 1636, 1535, 1454, 1350, 1240, 1169, 1038, 973, 841, 807, 791, 697 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.15 (d, *J* = 7.6 Hz, 1H), 7.95 (d, *J* = 8.2 Hz, 1H), 7.78 (d, *J* = 8.2 Hz, 1H), 7.33 – 7.27 (m, 6H), 7.2 – 7.15 (m, 4H), 6.95 (dd, *J* = 15.5, 5.0 Hz, 1H), 6.87 (d, *J* = 6.3 Hz, 2H), 5.14 (s, 1H), 5.02 (s, 2H), 4.94 (s, 1H), 4.52 (q, *J* = 6.1 Hz, 1H), 4.31 (q, *J* = 8.1 Hz, 2H), 3.89 (t, *J* = 8.1 Hz, 1H), 3.84 (s, 3H), 3.42 (dd, *J* = 13.7, 5.0 Hz, 1H), 3.03 (d, *J* = 11.8 Hz, 1H), 1.70 (s, 1H), 1.58 (dd, *J* = 12.7, 6.3 Hz, 2H), 1.46 (dt, *J* = 14.1, 7.2 Hz, 5H), 1.21 (d, *J* = 7.0 Hz, 3H), 1.09 (dt, *J* = 15.0, 8.0 Hz, 1H), 0.89 – 0.76 (m, 18H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 177.9, 171.4, 171.0, 170.9, 169.4, 163.7, 155.9, 149.6, 137.0, 134.2, 129.3, 128.2, 128.0, 127.6, 127.5, 126.7, 121.0, 94.9, 65.2, 59.1, 58.9, 50.8, 45.2, 41.1, 40.6, 36.3, 33.9, 24.3, 24.1, 24.0, 23.0, 21.6, 21.4,

19.4, 15.2, 10.8. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{43}H_{59}N_5O_8Na^+$, 796.4256; found, 796.4255.

*(2S,3S)-2-Amino-N-((5S,8S,11S)-8-isobutyl-3,5,13-trimethyl-4,7,10-trioxo-2-oxa-3,6,9-triazatetradecan-11-yl)-3-methylpentanamide (**36**).* A three-neck flask was charged with compound **30** (380 mg, 1.00 mmol), Pd/C (200 mg) and methanol (25 mL). The resulting suspension was stirred for 2 h in hydrogen atmosphere. The reaction mixture was filtered with diatomaceous earth. The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (dichloromethane: methanol=20: 1) to afford desired compound **36** as a white solid (864 mg, 85%). $[\alpha]_D^{20} = -62$, (c = 1.0, MeOH). m. p. 189 °C. v_{max}(KBr): 3382, 3269, 3083, 2958, 2934, 2872, 1631, 1550, 1466, 1369, 1244, 1174, 1057, 986, 955 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 8.15 – 7.86 (m, 3H), 4.78 – 4.57 (m, 1H), 4.34 (dt, *J* = 15.8, 7.8 Hz, 2H), 3.72 (s, 3H), 3.13 (d, *J* = 5.1 Hz, 1H), 3.09 (s, 3H), 1.89 (s, 1H), 1.59 (dt, *J* = 12.0, 6.6 Hz, 3H), 1.42 (q, *J* = 6.5 Hz, 5H), 1.23 (s, 1H), 1.18 (d, *J* = 7.1 Hz, 3H), 1.06 (dt, *J* = 14.6, 7.7 Hz, 1H), 0.90 – 0.78 (m, 18H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.1, 172.1, 171.6, 171.5, 61.1, 58.6, 50.6, 50.4, 44.6, 41.1, 40.7, 38.0, 24.1, 24.0, 23.7, 23.1, 23.0, 21.7, 21.4, 21.2, 16.9, 15.4, 11.5. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{23}H_{45}N_5O_5Na^+$, 494.3313; found, 494.3312.

*(2S,3S)-N-((5S,8S,11S)-8-Isobutyl-3,5,13-trimethyl-4,7,10-trioxo-2-oxa-3,6,9-triazatetradecan-11-yl)-2-(3-(4-methoxyphenyl)ureido)-3-methylpentanamide (**37a**).* A round-bottom flask was charged with compound **36** (300 mg, 0.64 mmol), TEA (64.8 mg, 0.64) and THF (7 mL). 4-Methoxyphenyl isocyanate (79.1 mg, 0.53 mmol) was added. The resulting solution was stirred overnight. The resulting mixture washed with 1 N hydrochloric acid solution, and extracted by ethyl acetate (3 × 20 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (dichloromethane: methanol=60: 1) to afford desired compound **37a** as a white solid (292 mg, 89%). $[\alpha]_D^{20} = -79$, (c = 1.0, MeOH). m. p. 220 °C. v_{max} (KBr): 3290, 3078, 2960, 2935, 1637, 1548, 1510, 1281, 1242, 1176, 1038, 991, 829, 767 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 8.46 (s, 1H), 8.11 (d, *J* = 8.1 Hz, 1H), 7.99 (d, *J* = 7.0 Hz, 1H), 7.85 (d, *J* = 8.3 Hz, 1H), 7.26 (d, *J* = 8.2 Hz, 2H), 6.80 (d, *J* = 8.2 Hz, 2H), 6.21 (d, *J* = 8.4 Hz, 1H), 4.66 (s, 1H), 4.33 (s, 2H), 4.16 (t, *J* = 6.8 Hz, 1H), 3.72 (s, 3H), 3.68 (s, 3H), 3.09 (s, 3H), 1.68 (s, 1H), 1.63 – 1.55 (m, 2H), 1.49 – 1.37 (m, 4H), 1.23 (s, 1H), 1.18 (d, *J* = 7.0 Hz, 3H), 1.03 (dt, *J* = 13.9, 7.9 Hz, 1H), 0.90 – 0.79 (m, 18H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.5, 171.4, 155.0, 153.8, 133.5, 119.0, 113.9, 61.1, 56.7, 55.1, 50.9, 50.4, 44.5, 40.8, 40.5, 37.8, 31.2, 24.1, 23.9, 23.1, 22.9, 21.6, 21.4, 16.9, 15.3, 11.4. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{31}H_{52}N_6O_7Na^+$, 643.3790; found, 643.3788.

*(2S,3S)-N-((5S,8S,11S)-8-isobutyl-3,5,13-trimethyl-4,7,10-trioxo-2-oxa-3,6,9-triazatetradecan-11-yl)-3-methyl-2-(2-(3-(trifluoromethyl)phenyl)acetamido)pentanamide (**37b**).* A round-bottom flask was charged with compound **36** (200 mg, 0.42mmol), *m*-(trifluoromethyl)phenylacetic acid (260 mg, 1.27 mmol), DIPEA (326 mg, 2.52 mmol), HATU (483 mg, 1.27 mmol) and DMF (5 mL). The resulting suspension was cooled to 0 °C and was stirred overnight. The reaction mixture was washed with 1 N hydrochloric acid solution, saturated sodium carbonate solution, and extracted by ethyl acetate. The combined organic phase were dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo.* The residue was purified by flash column chromatography on silica gel

(petroleum ether: ethyl acetate= 3:1) to afford desired compound **37b** as a white solid (229 mg, 83%). $[\alpha]_D^{20}$ = – 62.6, (c = 1.0, MeOH). m. p. 230 °C. ν_{max} (KBr): 3283, 3078, 2961, 2930, 2874, 1635, 1548, 1452, 1331, 1251, 1165, 1125, 1076, 995, 785 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.27 (d, *J* = 8.8 Hz, 1H), 8.05 (dd, *J* = 18.8, 7.8 Hz, 2H), 7.79 (d, *J* = 8.4 Hz, 1H), 7.63 (s, 1H), 7.59 – 7.49 (m, 3H), 4.71 – 4.60 (m, 1H), 4.31 (p, *J* = 7.8 Hz, 2H), 4.19 (t, *J* = 8.2 Hz, 1H), 3.72 (s, 3H), 3.67 – 3.53 (m, 2H), 3.09 (s, 3H), 1.71 (q, *J* = 6.6 Hz, 1H), 1.55 (ddq, *J* = 32.8, 12.8, 6.4 Hz, 2H), 1.40 (t, *J* = 7.0 Hz, 4H), 1.23 (s, 1H), 1.17 (d, *J* = 7.1 Hz, 3H), 1.04 (dt, *J* = 13.8, 8.0 Hz, 1H), 0.88 – 0.73 (m, 18H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.4, 170.7, 169.3, 138.1, 133.1, 129.1, 125.4, 123.0, 99.5, 61.1, 56.6, 50.9, 50.4, 44.5, 41.5, 40.9, 40.5, 36.7, 24.2, 24.0, 23.9, 23.2, 22.9, 21.5, 21.4, 16.9, 15.1, 10.8. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{32}H_{50}F_3N_5O_6Na^+$, 680.3605; found, 680.3603.

*N-((5S,8S,11S,14S,15S)-8,11-Diisobutyl-3,5,15-trimethyl-4,7,10,13-tetraoxo-2-oxa-3,6,9,12-tetraazaheptadecan-14-yl)pent-4-ynamide (**37c**).* The titled compound **37c** was obtained following the procedure described for **37b**. Flash column chromatography (petroleum ether: ethyl acetate= 3:1). 303 mg, 86%, as a white solid. $[\alpha]_D^{20}$ = – 64, (c = 1.0, MeOH). m. p. 170 °C. ν_{max}(KBr) 3290, 3080, 2961, 2935, 2874, 2376, 1637, 1545, 1464, 1171, 1092 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.98 (dt, *J* = 14.5, 7.8 Hz, 3H), 7.79 (d, *J* = 8.4 Hz, 1H), 4.69 – 4.61 (m, 1H), 4.31 (p, *J* = 8.3 Hz, 2H), 4.19 (t, *J* = 8.0 Hz, 1H), 3.72 (s, 3H), 3.09 (s, 3H), 2.72 (s, 1H), 2.41 – 2.28 (m, 4H), 1.68 (dt, *J* = 10.3, 5.1 Hz, 1H), 1.58 (dq, *J* = 13.2, 6.7 Hz, 2H), 1.46 – 1.38 (m, 4H), 1.23 (s, 1H), 1.17 (d, *J* = 7.1 Hz, 3H), 1.07 (dt, *J* = 13.6, 8.0 Hz, 1H), 0.88 – 0.77 (m, 18H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.5, 170.8, 170.3, 83.7, 71.2, 61.1, 56.7, 50.9, 50.4, 44.6, 40.8, 40.6, 36.6, 34.0, 24.3, 24.1, 23.9, 23.2, 23.0, 21.6, 21.4, 16.9, 15.2, 14.3, 11.0. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{28}H_{49}N_5O_6Na^+$, 574.3575; found, 574.3575.

*Aldehyde **38a-c**.*

A three-neck flask was charged with corresponding compound **37a-c** (1 eq.) and dry THF. resulting suspension cooled to – 20 °C in a nitrogen atmosphere. LAH (1.2 eq) was added and the reaction mixture was stirred 30 min. the reaction mixture was quenched with ethyl acetate. The reaction mixture was poured into 0.1 N hydrochloric acid solution. The mixture was separated and the aqueous phase was extracted with ethyl acetate. The combined organic layers was combined and dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo* to afford corresponding aldehyde, which could be used directly without any further purification.

*Diethyl (S)-(2-(2-isopropyl-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-2-oxoethyl)phosphonate (**39**).* A round-bottom flask was charged with (*S*)-1-(2-bromoacetyl)-5-isopropyl-4-methoxy-1,5-dihydro-2*H*-pyrrol-2-one (600 mg, 2.2 mmol) and triethyl phosphite (1.8 g, 11 mmol). The resulting solution was stirred overnight in 55 °C. After the substrate was completely consumed (monitored by TLC analysis), the reaction mixture was concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (petroleum ether: ethyl acetate= 1:1 to dichloromethane: methanol=15: 1) to afford phosphonate ester **39** as a pale yellow oil (540 mg, 74%). $[\alpha]_D^{20}$ = +65.9, (c = 1.0, MeOH). ν_{max}(KBr): 3109, 2975, 2931, 1736, 1692, 1616, 1435, 1352, 1331, 1221, 1100, 1005, 970 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.05 (s, 1H), 4.55 (d, *J* = 2.5 Hz, 1H), 4.18 – 4.09 (m, 4H), 4.06 (dd, *J* = 8.6, 1.5 Hz, 1H), 3.82 (d, *J* = 1.4 Hz, 3H), 3.56 (ddd, *J* = 21.8, 14.0, 1.4 Hz, 1H), 2.51 (pd, *J* = 7.0, 2.3 Hz,

1H), 1.28 (td, *J* = 7.1, 2.8 Hz, 6H), 1.06 (d, *J* = 7.1 Hz, 3H), 0.73 (d, *J* = 6.9 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) *δ* 179.7, 170.1, 164.1, 94.4, 64.1, 62.6, 62.4, 58.6, 36.5, 35.2, 28.7, 18.7, 16.4, 15.4. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{14}H_{24}NO_6PNa^+$, 356.1233; found, 356.1232.

*(S)-N-((S,E)-5-((S)-2-isopropyl-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-5-oxopent-3-en-2-yl)-2-((S)-2-((2S,3S)-2-(3-(4-methoxyphenyl)ureido)-3-methylpentanamido)-4-methylpentanamido)-4-methylpentanamide (**40a**).* A three-neck flask was charged with phosphonate ester **39** (142 mg, 0.43 mmol) and dry THF (4 mL). The resulting solution cooled to – 78 °C in a nitrogen atmosphere. LiHMDS (0.43 mL, 0.43 mmol) was added dropwise. The reaction mixture was stirred for 15 min. A solution of aldehyde **38a** (160 mg, 0.28 mmol, in THF 2 mL) was then added dropwise over 5 min. The resulting solution was stirred for further 3 h. After the substrate was completely consumed (monitored by TLC analysis), the reaction mixture was quenched with H_2O, and extracted by ethyl acetate. The combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (dichloromethane: methanol=50: 1 to 20: 1) to afford desired compound **40a** as a white solid (108 mg, 52%). $[\alpha]_D^{20}$ = – 4.1, (c = 1.0, MeOH). m. p. 244 °C. ν_{max}(KBr): 3277, 2961, 2932, 1729, 1634, 1548, 1465, 1326, 1247, 1169, 1026, 993, 828, 804, 713, 661 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) *δ* 8.50 (d, *J* = 4.8 Hz, 1H), 8.09 (dd, *J* = 14.0, 8.0 Hz, 2H), 7.81 (d, *J* = 8.3 Hz, 1H), 7.32 – 7.22 (m, 3H), 6.88 – 6.77 (m, 3H), 6.29 – 6.17 (m, 1H), 5.38 (s, 1H), 4.59 (d, *J* = 2.5 Hz, 1H), 4.55 – 4.46 (m, 1H), 4.32 (p, *J* = 7.4 Hz, 2H), 4.13 (dd, *J* = 8.5, 5.9 Hz, 1H), 3.85 (s, 3H), 3.68 (s, 3H), 1.69 (d, *J* = 6.2 Hz, 1H), 1.58 (dt, *J* = 13.6, 6.7 Hz, 2H), 1.51 – 1.40 (m, 5H), 1.23 (s, 2H), 1.19 (d, *J* = 7.0 Hz, 3H), 1.04 (d, *J* = 7.2 Hz, 3H), 0.88 – 0.79 (m, 18H), 0.66 (dd, *J* = 6.9, 3.6 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) *δ* 179.5, 171.6, 171.5, 171.2, 170.0, 163.4, 155.1, 153.8, 149.5, 133.5, 121.2, 119.0, 113.9, 94.7, 63.0, 59.1, 56.9, 55.1, 51.0, 50.8, 45.2, 41.1, 40.5, 37.7, 31.3, 29.0, 28.1, 24.1, 23.1, 22.9, 21.6, 21.4, 19.5, 18.6, 15.4, 15.1, 11.4. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{39}H_{60}N_6O_8Na^+$, 763.4365; found, 763.4363.

*(S)-N-((S,E)-5-((S)-2-isopropyl-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-5-oxopent-3-en-2-yl)-4-methyl-2-((S)-4-methyl-2-((2S,3S)-3-methyl-2-(2-(3-(trifluoromethyl)phenyl)acetamido)pentanamido)pentanamido)pentanamide (**40b**).* The titled compound **40b** was obtained following the procedure described for **40a**. Flash column chromatography (dichloromethane: methanol=50: 1 to 15: 1). 95 mg, 47%, as a white solid. $[\alpha]_D^{20}$ = – 13.6, (c = 1.0, MeOH). m. p. 192 °C. ν_{max}(KBr): 3282, 3079, 2963, 2934, 2875, 1730, 1636, 1546, 1452, 1330, 1164, 1124, 807, 786, 701 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) *δ* 8.27 (d, *J* = 8.8 Hz, 1H), 8.09 (dd, *J* = 21.4, 8.0 Hz, 2H), 7.76 (dd, *J* = 18.0, 8.4 Hz, 1H), 7.63 (s, 1H), 7.60 – 7.50 (m, 3H), 7.27 (d, *J* = 15.5 Hz, 1H), 6.84 (dd, *J* = 15.5, 4.9 Hz, 1H), 5.38 (s, 1H), 4.59 (d, *J* = 2.5 Hz, 1H), 4.50 (q, *J* = 6.7 Hz, 1H), 4.30 (t, *J* = 7.6 Hz, 2H), 4.18 (t, *J* = 8.3 Hz, 1H), 3.85 (s, 3H), 3.68 – 3.53 (m, 2H), 2.47 – 2.39 (m, 1H), 1.77 – 1.67 (m, 1H), 1.63 – 1.55 (m, 1H), 1.49 (dt, *J* = 14.8, 7.0 Hz, 3H), 1.41 (t, *J* = 7.4 Hz, 3H), 1.23 (s, 1H), 1.18 (d, *J* = 6.8 Hz, 3H), 1.04 (d, *J* = 7.1 Hz, 3H), 0.89 – 0.75 (m, 18H), 0.67 (d, *J* = 6.9 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) *δ* 179.5, 171.3, 171.1, 170.7, 169.9, 169.4, 163.4, 149.5, 138.1, 133.1, 129.1, 128.7, 125.4, 123.0, 121.2, 94.7, 63.0, 59.1, 56.7, 50.9, 50.7, 45.2, 41.5, 41.2, 40.5, 36.6, 28.1, 24.3, 24.1, 23.1, 22.9, 21.6, 21.5, 19.5, 18.5, 15.2, 15.1, 10.8. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{40}H_{58}F_3N_5O_7Na^+$, 800.4181; found, 800.4180.

*N-((2S,3S)-1-(((S)-1-(((S)-1-(((S,E)-5-((S)-2-isopropyl-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-5-oxopent-3-en-2-yl)amino)-4-methyl-1-oxopentan-2-yl)amino)-4-methyl-1-oxopentan-2-yl)amino)-3-methyl-1-oxopentan-2-yl)pent-4-ynamide (**40c**).* The titled compound **40c** was obtained following the procedure described for **40a**. Flash column chromatography (dichloromethane: methanol=80: 1 to 20: 1). 85 mg, 42%, as a yellow solid. $[\alpha]_D^{20}$ = – 0.8, (c = 1.0, MeOH). m. p. 133 °C. ν_{max}(KBr): 3286, 3078, 2961, 2933, 2875, 2119 1727, 1633, 1548, 1457, 1174, 993, 962 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.89 (dd, *J* = 8.0, 4.3 Hz, 1H), 7.81 (dd, *J* = 8.5, 4.7 Hz, 2H), 7.56 (dd, *J* = 8.7, 4.6 Hz, 1H), 7.04 (dd, *J* = 15.3, 3.9 Hz, 1H), 6.61 (dt, *J* = 15.5, 4.6 Hz, 1H), 5.15 (d, *J* = 4.1 Hz, 1H), 4.36 (d, *J* = 3.1 Hz, 1H), 4.27 (p, *J* = 6.0, 5.4 Hz, 1H), 4.12 – 4.02 (m, 2H), 3.94 (td, *J* = 8.1, 3.9 Hz, 1H), 3.62 (d, *J* = 3.7 Hz, 3H), 2.50 (dd, *J* = 4.9, 2.6 Hz, 1H), 2.30 – 2.25 (m, 1H), 2.23 – 2.06 (m, 5H), 1.47 (d, *J* = 9.4 Hz, 1H), 1.33 (dd, *J* = 12.2, 6.0 Hz, 2H), 1.29 – 1.16 (m, 4H), 0.97 (dq, *J* = 12.2, 4.9, 4.5 Hz, 4H), 0.82 (dd, *J* = 7.3, 3.9 Hz, 3H), 0.67 – 0.53 (m, 18H), 0.44 (dd, *J* = 7.2, 3.8 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 179.5, 171.4, 171.1, 170.9, 170.4, 170.0, 163.4, 149.5, 121.2, 94.7, 83.7, 71.2, 63.0, 59.1, 56.9, 51.0, 50.8, 45.2, 41.1, 40.5, 36.5, 34.0, 28.1, 24.3, 24.1, 23.1, 22.9, 21.6, 21.4, 19.5, 18.6, 15.3, 15.1, 14.3, 11.0. HRMS-ESI (m/z): $[M+Na]^+$ calcd. for $C_{36}H_{57}N_5O_7Na^+$, 694.4150; found, 694.4148.

Cell lines and cell culture

Human breast cancer cell lines MDA-MB-231 (ATCC CRM-HTB-26), MCF-7 (ATCC HTB-22^{TM}), human melanoma cell line A375 (ATCC CRL-1619), human pancreatic cancer cell line PANC-1 (ATCC CRL-1469), human lung adenocarcinoma cell line A549 (ATCC CRM-CCL-185) and mouse breast cancer cell line 4T1 (ATCC CRL-2539), mouse melanoma cell line B16-F10 (ATCC CRL-6475) , were cultured in 1640 medium supplemented with 10% fetal bovine serum (FBS, Biological Industries (BI), 04-001-1A/B), 100 units/ml penicillin G, 0.1 mg/ml streptomycin (life science, P1400) at 37 °C in the presence of 5% CO_2.

Cell cytotoxicity assay

MTT assays were used to assess the drug sensitivity of different cancer cell lines. Cells were seeded in 96-well plates at 5.0 × 10^3 cells/well for human cancer cell lines MDA-MB-231, MCF-7, A375, A549 and PANC-1, and 3.0 × 10^3 cells/well for mouse cancer cell line 4T1 and B16-F10 in a final volume of 100 µl. And then compounds were added at different concentrations after 24h post-plating. After 48 h of drug incubation, 15 µl of MTT (5 mg/ml, MedChemExpress (MCE, HY-15924) solution was added to each well and incubation was continued for another 3-4 h. Supernatant was removed and formazan crystals were dissolved in 100 µl of dimethyl sulfoxide (DMSO). Optical density (OD) of formazan concentration was read at 490 and 570 nm with THERMO FISHER Multiskan FC. The concentrations required to inhibit growth by 50% (IC_{50}) were calculated with GraphPad Prizm7 as previous description.

Colony formation assay

For clonogenic assay, 0.4×10^3 A375 cells and 0.3×10^3 B16-F10 cells respectively diluted in 2 ml culture medium were plated into 6-well culture plates. After a 24-h incubation, compound was added at indicated concentrations, and the plates were incubated at 37°C with 5% CO_2 for 7 days. During incubation, culture medium with compound was replaced twice. Finally, cell colonies were stained using 0.05% crystal violet (Amresco) and clones containing more than 50 cells were

counted

Apoptosis Assay

A375 and B16-F10 cancer cells (5× 10^5 cells/mL and 3× 10^5 cells/mL respectively) were plated into six-well culture plates and treated with compounds (35b) at indicated concentration for 48 h. The treated cells were then harvested by trypsinization and washed twice with cold PBS. Cell apoptosis was detected using an Annexin V-FITC / PI Apoptosis Detection kit (Beyotime, C1063) by BD flow cytometer.

Wound healing assay

The wound healing assay was normally used to study cell migration in vitro. A total of 1.5x10^5 A375 cells and 1.0x10^5 B16-F10 cells were seeded in 48-well culture plates and incubated overnight until confluent. A (yellow) pipette tip was used to make a straight scratch, and the suspension cells were washed off gently with PBS. Next, fresh medium supplemented with compound at indicated concentration was added and images of the scratch were acquired as control. Finally, images of the same location were obtained at 24h and 48h respectively.

Cell migration and invasion assay

Cell migration was assessed using transwell chamber (Corning, 3422) in 24-well plates. 200 μL of serum-free medium containing compound and 2×10^5 A375 or B16-F10 cells were plated into the upper chambers. A total volume of 0.6 ml medium supplemented with 10% FBS was then added to the wells as a chemoattractant. Cells were incubated for 24h at 37°C in 5% CO2.

Matrigel Invasion Chambers was used to study the ability of cell invasion. Briefly, matrigel was diluted at 1:3 ratios with medium firstly and 50 uL matrigel-medium mixture was added into upper chambers. The upper chambers with matrigel were incubated for 2h at 37°C in 5% CO2 until solidification. Then 200 μL of serum-free medium containing compound and 1×10^5 cells A375 or B16-F10 cells were plated into the upper chambers with matrigel treatment. A volume of 0.6 ml of medium supplemented with 10% FBS was then added to the wells as a chemoattractant. Cells were incubated for another 24 h at 37°C in 5% CO2.

Incubation for 24h, cells located in upper membrane were removed gently and the bottom of the membrane were fixed and stained with 0.1% crystal violet (Amresco). The migrated cells were counted from five different microscopic fields and the average number was calculated.

Tumor spheres assay

For tumor sphere formation assays, A375 and B16-F10 cells were pretreated with compound at indicated concentration for 48h, then cells were harvested and 500 cells per mL were plated into 24-well ultra-low attachment plate (Corning) in tumorsphere medium (20 ng/ml epidermal growth factor, 10 ng/mL basic fibroblast growth factor, 5 μg/mL insulin, 0.4% Bovine Serum Albumin and Dulbecco's Modified Eagle Medium/F12). The upper and lower edges of the 24-well plate were sealed with 1 X PBS to avoid evaporation of medium and the plate was placed in an incubator with 5% CO2 at 37 °C for 7 days. After one-week incubation, tumorspheres which size were large than 50 micrometers were counted.

ALDH activity assay

ALDH activity assay was done using the ALDEFLUOR kit as the manufacturer's instructions (Stem cell Technologies, 01700). $5x10^5$ A375 and $3x10^5$ B16-F10 cells were plated in 2mL in a 6-well culture plate and cells were cultured in an incubator with 5% CO_2 at 37 °C for 24h. Next, cells were treated with compound at indicated concentrations for 48h, and then cells were harvested. Finally, ALDH positive and negative cells were FACS sorted using BD FACSVerse.

$CD133^+$ cells isolated by Flow cytometry

$5x10^5$ A375 and 3x105B16-F10 cells were plated in 2ml in a 6-well culture plate and cells were cultured in an incubator with 5% CO2 at 37 °C for 24h. Next, cells were treated with compound at indicated concentrations for 48h, and then cells were harvested and washed twice in PBS. $1X10^6$ cells were incubated for 60 min at 4 °C in PBS with anti-CD133-APC antibodies (eBioscience Company, USA), using the manufacturer's suggested concentrations. Finally, the cells were washed twice in PBS, and then was analyzed with BD FACSVerse.

Western blot analysis

A375 (5×10^5 per well) and B16-F10 (3×10^5 per well) were seeded in 6-well culture plates, treated with compound at indicated concentrations for 48h and lysed in RIPA lysis buffer supplemented with protease inhibitors cocktail / PMSF and dephosphorylase inhibitor NaF . The protein concentration was measured with the BCA Protein Assay kit (Beyotime Biotechnology, P0012). Equal amounts of proteins were electrophoresed by SDS-PAGE (8%-15%) under denaturing conditions and transferred onto the PDVF membranes (Millipore Corporation, IPVH00010). Membranes were blocked in 5% non-fat milk (BD Difco, 232100) and then incubated with primary antibodies. After being washed, the membranes were incubated with secondary antibodies, and detected by Luminescent Image Analyzer LSA 4000 (GE, Fairfield, CO, USA). Antibodies used in this study were: anti-cleaved PARP (#9541), anti-cleaved caspase-3 (#9661), anti-Bax (#2772), anti-β-actin (#3700), anti-p-STAT3 (#9145, CST, Beverly, MA, USA), anti-STAT3 (10253-2-AP), anti-P53(10442-1-AP), anti-c-Myc (10828-1-AP), anti-β-catenin (51067-2-AP, Proteintech, North America, HQ, USA)

Animal study

The animal studies were approved by the Institutional Review Board of Nankai University. All animal studies were conducted according to protocols approved by the Animal Ethics Committee of Nankai University. A total volume of 0.1 mL PBS containing 5×10^5 B16-F10 cells was injected subcutaneously into six-week-old C57BL/6 mice. All mice were kept in specific pathogen-free (SPF) environment. When the tumor volume reached about 50 mm^3, the mice were randomly divided to vehicle control, **35b** (15 mg/kg/d every day, ip, n = 6 for each group) and were treated as above dose. Tumor volume and mice weight were measured each day after the initiation of the treatment. Mice were executed and tumors were harvested on day 24.

Statistical analysis

All statistical analyses were conducted using SPSS 19.0 software for Windows (Chicago, IL). Statistically significant differences were determined by two-way ANOVA, and *P* values less than 0.05 were considered statistically significant in all cases.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version. These data include NMR spectrums of the most compounds described in this article.

Conflicts of interest

The authors declare no competing financial interest.

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A Novel Anti-Cancer Stem Cells Compound Optimized from the Natural Symplostatin 4 Scaffold Inhibits Wnt/β-Catenin Signaling Pathway

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Highlights：

Cancer Stem Cells

Total Synthesis

Symplostatin 4

Depsipeptides